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Highlights

- Quinoa and ice plant are resilient plants covered with epidermal bladder cells (EBCs)
- EBCs constitute an effective barrier toward arthropod herbivores and a phytopathogen
- In contrast, EBCs hardly contribute to the abiotic stress tolerance of these plants
- EBC density therefore emerges as a breeding target for biotic stress resistance

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In brief

The plants quinoa and ice plant are covered with modified trichomes called epidermal bladder cells (EBCs). Moog et al. challenge current models that link these to abiotic stress tolerance and instead show that EBCs form a barrier against herbivores and a phytopathogen.

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Epidermal bladder cells as a herbivore defense mechanism

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SUMMARY

The aerial surfaces of quinoa (*Chenopodium quinoa*) and common ice plant (*Mesembryanthemum crystallinum*) are covered with a layer of epidermal bladder cells (EBCs), which are modified non-glandular trichomes previously considered to be key to the extreme salt and drought tolerance of these plants. Here, however, we find that EBCs of these plants play only minor roles, if any, in abiotic stress tolerance and in fact are detrimental under conditions of water deficit. We report that EBCs instead function as deterrents to a broad range of generalist arthropod herbivores, through their combined function of forming both a chemical and a physical barrier, and they also serve a protective function against a phytopathogen. Our study overturns current models that link EBCs to salt and drought tolerance and assigns new functions to these structures that might provide novel possibilities for protecting crops from arthropod pests.

INTRODUCTION

The escalating abiotic and biotic stresses due to climate change necessitate increased irrigation and pesticide use, counter to sustainability goals.[1,2] Consequently, resilient crops like quinoa (*Chenopodium quinoa* Willd.) have garnered attention.[3–6] However, global quinoa expansion faces challenges, such as exposure to unfamiliar arthropod herbivores and phytopathogens.[7,8]

A striking morphological feature of quinoa is its specialized non-glandular trichomes, called epidermal bladder cells (EBCs), also found in the common ice plant (*Mesembryanthemum crystallinum* L.), both within the order Caryophyllales. For a long time believed to aid in abiotic stress tolerance, these EBCs have been extensively studied for their role in salt tolerance as potential salt reservoirs.[9–16] Notably, an EBC-free (*ebcf*) quinoa mutant proved as salt tolerant as the wild type (WT).[17] Moreover, EBCs in quinoa preferentially accumulate K^+ over Na^+, suggesting no direct involvement in managing Na^+ overload.[17] In ice plant, Lüttge et al.[15] found EBCs and leaf lamina cells reached Cl^-, Na^+, and K^+ equilibrium. Subsequently, Agarie et al.[16] concluded that EBCs in WT plants contained less Na^+ and Cl^- than the underlying cells.

Metabolomic analysis of EBCs from abiotic stress-exposed quinoa showed minimal changes in cellular contents.[18] Instead, EBCs contained plant defense compounds against herbivores, like saponins and oxalic acid,[18] pointing toward a largely overlooked role in deterring herbivores.[19] Additionally, EBCs are proposed to mitigate water loss and UV damage in ice plant[13,20,21] and quinoa.[6,11,22]

To elucidate the physiological functions of EBCs, we conducted a genetic study using quinoa and ice plant mutants with reduced EBC numbers.[16,17,22] Our findings indicate that EBCs do not enhance abiotic stress tolerance and are even detrimental under water deficit conditions. Rather, they seem to deter generalist herbivores. Optimizing quinoa EBC densities could facilitate crop adaptation beyond the Andean region, thus expanding cultivation.

RESULTS

EBCs cannot reduce transpiration

It has been hypothesized that EBCs may contribute to quinoa's water deficit tolerance through reducing transpiration by forming a secondary epidermis and by functioning as water reservoirs.[6] First, we failed to detect any differences in well-watered WT and *ebcf* plants with respect to growth performance (Figure 1A), relative water content (RWC), plant biomass (dry weight [DW]), total leaf area, chlorophyll content, and F_v/F_m (Figures S1A–S1E). Furthermore, we were not able to observe any differences in transpiration rates of quinoa WT and plants lacking EBCs, be it in *ebcf* or WT plants from which we mechanically removed EBCs by gently brushing their surface with a cosmetic brush[12] (Figure 1B). Thus, WT and *ebcf* plants seem to grow equally well in the absence of water stress,[17] and EBCs do not seem to inhibit transpiration.

Quinoa mutants without EBCs show elevated water deficit tolerance as EBCs act as water sinks not sources

To test the possible contribution of EBCs as water reservoirs, we withheld water from 30-day-old quinoa plants that had been well

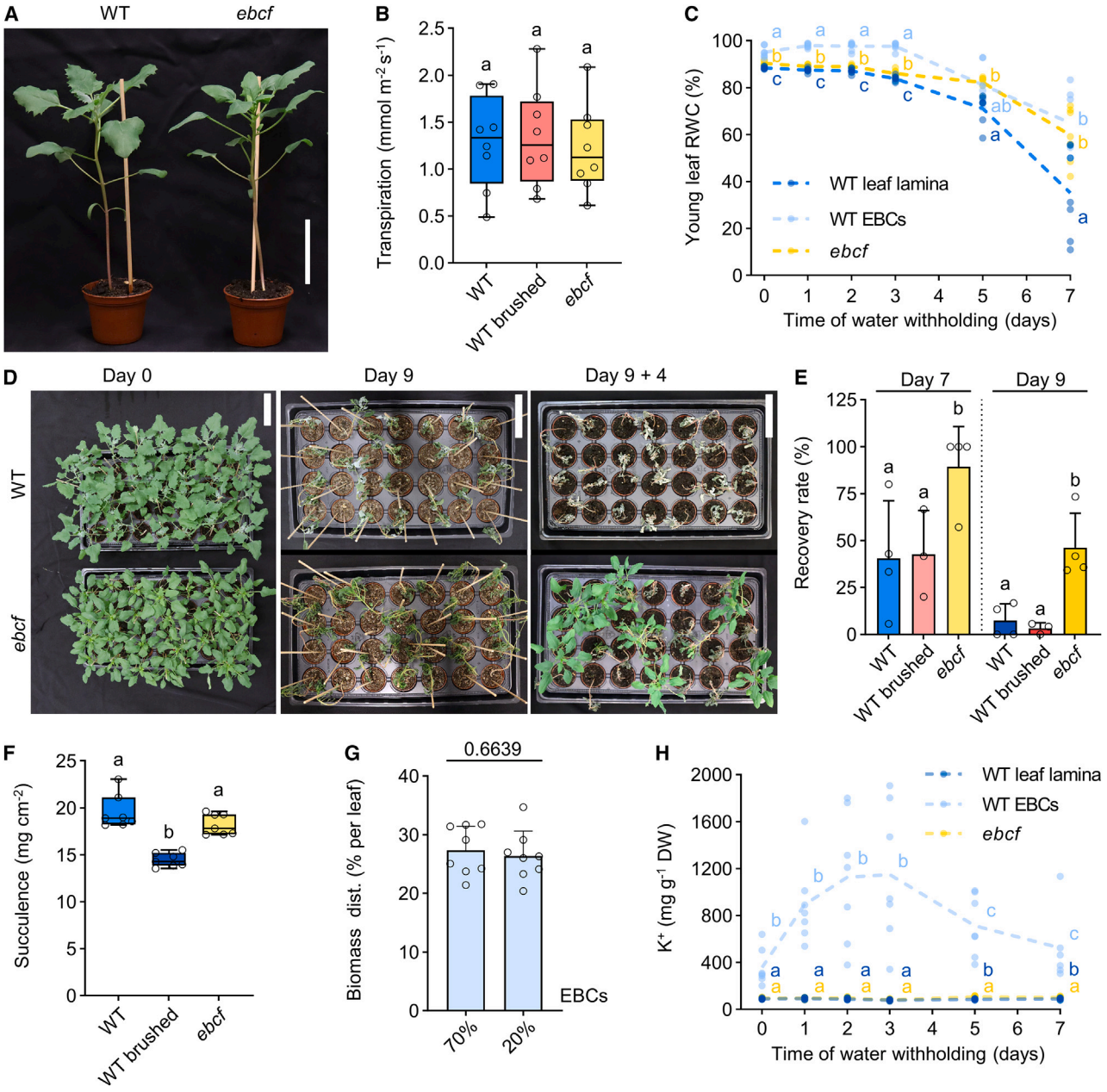

Figure 1. The *ebcf* mutant of quinoa is more drought tolerant than the wild type

(A) Representative photograph of 30-day-old wild-type (WT) and *ebcf* quinoa plants. Scale bars, 5 cm.

(B) Transpiration rates from young leaves of WT plants with EBCs intact or removed by brushing and from leaves of *ebcf* plants. Open circles represent individual values. The boxes extend from the 25th to the 75th percentiles, with horizontal lines indicating the medians ± SD and whiskers ranging from minimum to maximum values; n = 8. p values were obtained from a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Different letters indicate significant differences ($p < 0.05$).

(C) Changes in relative water content (RWC) of the leaf lamina and EBCs of young WT leaves (13th leaf) and of young *ebcf* leaves over a 7-day period of water withholding. Filled circles present individual values, connected by the means; n = 7 (n = 6 for WT EBCs day 7). p values were obtained from a mixed-effects model (restricted maximum likelihood, REML) followed by Tukey's multiple comparisons test (within individual time points). Different letters indicate significant differences ($p < 0.05$). See also Table S1.

(D) Representative photographs of 30-day-old wild-type (WT) and *ebcf* quinoa plants before (day 0) or after 9 days (day 9) of water deficit stress by water withholding and 4 days after rewatering (day 9 + 4). Scale bars, 10 cm.

(E) Recovery rates of WT plants with EBCs intact or removed 3 days prior to drought treatment by gentle brushing, as well as *ebcf* plants after 7 or 9 days of water withholding. Open circles represent individual values. Bars are means ± SD; n = 4 recovery experiments for WT and *ebcf* and n = 3 for WT brushed. p values were obtained from a two-way ANOVA followed by Tukey's multiple comparisons test (within individual time points). Different letters indicate significant differences ($p < 0.05$).

(legend continued on next page)

watered since germination. Following water withholding, RWCs decreased faster in WT compared with mutant plants (Figure S1A). Mature WT leaves (3rd leaf) with low EBC densities lost water at a lower rate than young leaves with high EBC densities (Figures 1C and S1F): young leaves lost 60% of their water, whereas old leaves lost only 50% (Table S1). In contrast, young and old *ebcf* leaves without EBCs lost water to the same degree (Table S1). After 9 days, the WT plants had dried out, whereas the *ebcf* plants remained green despite wilting (Figure 1D). After resumption of watering for 4 days (Figure 1D), only ≈10% of WT plants were still alive, whereas ≈50% of the *ebcf* plants had fully recovered. A shorter water-withholding period of 7 days followed by rewatering for 4 days led to recovery rates of ≈40% for WT plants but close to 100% for *ebcf* plants (Figure 1E). As the DW and leaf area of WT and *ebcf* did not differ, the succulence (weight per area) of WT and *ebcf* leaves was also identical (Figure 1F). However, as EBCs contribute ~25% of the leaf biomass (Figure 1G), the succulence of the WT leaf lamina (WT leaf with EBCs removed by brushing) was lower than that of the *ebcf* leaf lamina (Figure 1F).

Similarly, *rebc-2* quinoa plants, which have reduced numbers of EBCs and previously were shown to have aberrant shoot apices under various abiotic stress conditions,[22] also showed higher recovery rates than the corresponding WT plants after 8 days of water withholding and 4 days of rewatering (Figure S1G), although both had the same RWC before water withholding started (Figure S1H). Accordingly, succulence was strongly affected by the presence of EBCs (Figure S1I).

Whereas the leaf laminas of WT quinoa lost turgor during water stress, the EBCs apparently did not deflate. We quantified water loss by mechanically removing EBCs from young leaves and measuring RWCs in the EBCs versus the remaining leaf lamina. Contrary to the expectation of EBCs being water reservoirs, after 7 days of water withholding, not only was the RWC of the EBCs (~70%) much higher than that of WT leaf laminas (~30%) (Figure 1C) but the leaf laminas also lost water more rapidly, whereas the RWC of EBCs remained stable for a longer time span (Table S1). Compared with the recovery of WT plants whose EBCs had not been removed, the mechanical removal of the water-filled EBCs from WT plants through gentle brushing prior to water withholding did not decrease recovery rates after watering was resumed (Figure 1E). We observed no significant difference in growth between WT and *ebcf* plants supplied with sufficient water (water-holding capacity [WHC] of 70%) or subjected to water stress (WHC of 20%) (Figure S1J); furthermore, we failed to find differences in to what extend WT plants produced EBCs on their leaves when grown under conditions of sufficient or strongly reduced water availability (Figure 1G).

EBCs of quinoa accumulate K^+, which serves as an osmolyte to retain water.[17] We found that the K^+ contents in drought-stressed young leaf laminas of both WT and *ebcf* plants remained relatively low and constant (≈100 mg g^{-1} DW). In contrast, the K^+ content of EBCs was significantly higher (≈350 mg g^{-1} DW) before the onset of water withholding, tripled (≈1,150 mg g^{-1} DW) following water withholding for 3 days, and decreased under continued water withholding (Figure 1H).

These results suggest that EBCs act as a sink for water and withdraw water from the leaf lamina, as they take up K^+ to a greater extent than surrounding cells, especially under water shortage conditions. The transfer of K^+ and subsequently water into the EBCs decreases the succulence of the laminas of EBC-covered leaves. In contrast, leaves of mutant plants lacking EBCs have increased succulence and therefore retain water.

No influence of EBCs in the salt tolerance of quinoa could be identified

We re-evaluated the salt tolerance of *rebc-2* under controlled conditions in growth chambers, as we had previously observed that the *ebcf* mutant is as salt tolerant as the WT.[17] Although *rebc-2* exhibited an overall lower biomass compared with the WT, this was independent of the salt regime (Figure S1K). When we corrected for the relative reduction in DW, we observed no differences in salt tolerance between the WT and *rebc-2* (Figure S1L). These findings provide additional genetic evidence that EBCs do not contribute to the salt tolerance of quinoa.

EBCs of the ice plant do not significantly alter water deficit tolerance

We performed similar experiments in ice plants to determine the role of EBCs in water deficit tolerance using WT and *ebc-less* plants[16] subjected to continuous and progressive water deficit stress. We failed to detect differences in DW and RWC between WT and *ebc-less* plants when they were grown in soil with reduced WHC values (continuous water deficit) (Figures S2A and S2B).

To test the potential role of EBCs as water reservoirs in progressive water deficit tolerance, we withheld water from plants of both genotypes after an initial 5 weeks of growth under well-watered conditions and assessed their subsequent performance compared with plants maintained in well-watered conditions (Figure 2A). Water-stressed WT and *ebc-less* plants survived a 15-week period without watering and completed their life cycle (Figure 2A). Leaves present at the onset of water withholding progressively dried out and new leaves and inflorescences emerged (Figure 2A). Notably, the emerging leaves and

(F) Succulence of leaves from WT with intact EBCs, WT with EBCs removed, and *ebcf* presented in weight per area of well-watered plants. Open circles represent individual values. The boxes extend from the 25th to the 75th percentiles, with horizontal lines indicating the medians and whiskers ranging from minimum to maximum values; n = 7 leaves. p values were obtained from a one-way ANOVA followed by Tukey's multiple comparisons test. Different letters indicate significant differences ($p < 0.05$).
(G) Relative fresh weight biomass of EBCs of WT leaves from plants grown under water deficit conditions (20% soil water-holding capacity [WHC]) and well-watered conditions (70% WHC). Open circles represent individual values. Bars are means ± SD; n = 8. p value was obtained from an unpaired (two-tailed) t test.
(H) Changes in K^+ concentrations in WT leaf lamina and EBCs and from the *ebcf* mutant leaves over a 7-day period of water withholding. Filled circles present individual values, connected by the means; n = 7 (n = 6 for WT EBCs day 7). p values were obtained from a mixed-effects model (REML) followed by Tukey's multiple comparisons test comparing within individual time points. Different letters indicate significant differences ($p < 0.05$).
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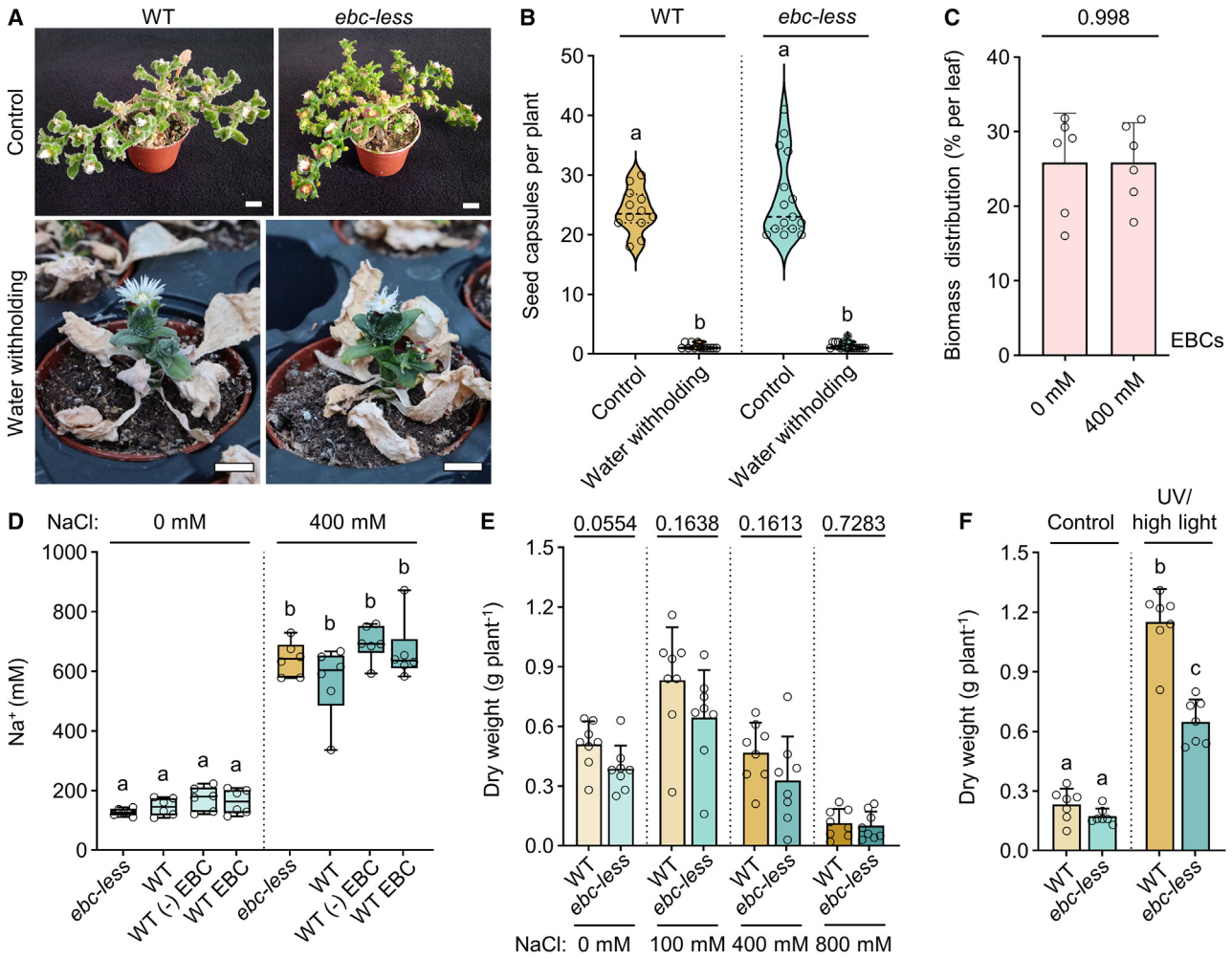

Figure 2. No differences in drought and salt tolerance can be detected between the WT and the *ebc-less* mutant of ice plants

(A) Representative photographs of well-watered (control) 23-week-old WT and *ebc-less* mutant plants and of 14-week-old plants that had not been watered (water withholding) for 9 weeks. Scale bars, 1 cm.

(B) Number of seed capsules produced by well-watered (control) and unirrigated (water withholding) WT and *ebc-less* plants. Open circles represent individual values. Violin plot with quartiles and median; n = 12 plants for WT, n = 15 plants for *ebc-less*. p values were obtained from a two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Different letters indicate significant differences ($p < 0.05$).

(C) Relative biomass of EBCs of WT leaves from plants watered with tap water (0 mM) or with 400 mM NaCl. Open circles represent individual values. Bars are means ± SD; n = 6. p value was obtained from an unpaired (two-tailed) t test.

(D) Na^+ concentration in young leaves of *ebc-less* and WT plants based on the water fraction of samples from plants watered with tap water (0 mM NaCl) and with 400 mM NaCl. Concentration was measured in additional leaves of the WT, which were separated into leaf lamina without EBCs (WT (-) EBC) and the EBC fraction (WT EBC). Open circles represent individual values. The boxes extend from the 25th to the 75th percentiles, with horizontal lines indicating the medians and whiskers ranging from minimum to maximum values; n = 6. p values were obtained from a two-way ANOVA followed by Tukey's multiple comparisons test. Different letters indicate significant differences ($p < 0.05$).

(E) Dry weight of 10-week-old WT and *ebc-less* plants treated with different NaCl concentrations for 5 weeks. Open circles represent individual values. Bars are means ± SD; n = 8. p values were obtained from multiple unpaired (two-tailed) t tests.

(F) Dry weight of 9-week-old WT and *ebc-less* ice plants grown at 180 μE (control) and 450 μmol m^{-2} s^{-1} with UV-B light of 32 μW/cm^2 for 4 weeks (UV/high light). Open circles represent individual values. Bars are means ± SD; n = 7. p values were obtained from a two-way ANOVA followed by Tukey's multiple comparisons test. Different letters indicate significant differences ($p < 0.05$).

This figure is related to Figures S2–S4.

inflorescences of WT plants were covered with EBCs during the drought period (Figure 2A). EBC density in *ebc-less* plants also increased as the plants matured, with EBCs being present on inflorescences of both well-watered and water-limited plants (Figure 2A). The number of seed capsules per plant was drastically lower in both genotypes under water withholding conditions (WT, from an average of 24.0–1.25 capsules per plant; *ebc-less*, from 26.3 to 1.4) (Figure 2B).

Thus, the exceptional water-withholding tolerance was not influenced by the presence of EBCs and, contrary to being water reservoirs, EBCs were produced even under severe water shortage.

Ice plant EBCs do not accumulate Na^+ to a higher extent than the underlying leaf

We previously showed that quinoa EBCs do not accumulate Na^+ in response to salt stress.[17] To measure EBC contents in ice plant, we punctured the cells with a small glass capillary and collected the watery contents. We sampled the remaining leaf patches to calculate the relative biomass of each leaf fraction. Although we did not collect EBC membranes or cell walls, the large volume of EBCs implies that cellular content weights give a good estimation of fresh weight (FW) biomass. We established that 25% of the leaf (FW) is derived from EBCs (Figure 2C). We measured no difference in relative biomass in plants grown under control conditions or 400 mM NaCl treatment (Figure 2C).

We determined the ion concentrations in the watery contents of EBCs and the underlying cells from WT as well as *ebc-less* mutant leaves. We calculated all concentrations based on the water fraction of the leaf to compare with the watery EBC contents we had collected. During salt treatment, the Na^+ concentration in entire leaves increased from 150 to about 600 mM in both WT and mutant plants (Figure 2D). However, Na^+ concentrations in EBCs and the underlying leaf did not significantly differ (Figure 2D). Cl^- concentration increased from around 200 to 600 mM in all samples when plants were treated with 400 mM NaCl (Figure S2C). By contrast, K^+ concentrations decreased in samples collected from salt-treated plants and were lowest in EBCs (Figure S2D). No differences in ion accumulation between leaf laminas of WT and *ebc-less* plants could be detected (Figures 2D, S2C, and S2D).

Together, these results confirm the previous observation that ice plant EBCs do not accumulate Na^+ and Cl^- to a higher extent than other leaf tissues.[15,16] More importantly, the ion concentrations in the leaf lamina are not affected by the presence of EBCs. Thus, ice plant EBCs do not seem to contribute to ion homeostasis in salt stress conditions.

EBCs of the ice plant do not significantly impact salt tolerance

The salt tolerance of the *ebc-less* ice plant mutant was previously assessed in hydroponic culture.[16] Here, ice plants grown in hydroponic culture showed a high variation in biomass acquisition, with their shoot DW ranging from 0.12 to 3.12 g in the WT and 0.38 to 3.5 g in mutant plants under control conditions (Figure S2E). Therefore, we failed to observe any effect from Na^+ and Cl^- treatments (Figures S2E and S2F).

Instead, we evaluated the performance of soil-grown WT and *ebc-less* plants after irrigation with water containing different salt concentrations: control conditions with no salt or mild (100 mM NaCl), intermediate (400 mM NaCl), or severe (800 mM NaCl) salt stress treatment (Figure S2G). No difference in growth between WT and *ebc-less* could be detected regardless of salt treatment (Figure 2E). Furthermore, following salt treatment, whole WT and *ebc-less* plants accumulated Na^+, K^+, and Cl^- to the same levels in their shoots (Figures S2H–S2J).

Thus, the *ebc-less* mutant, with greatly reduced EBC densities, is as salt tolerant as the WT, and ice plant EBCs do not play a significant role in aiding salt tolerance.

EBCs may protect ice plants under high-light/UV-B conditions

To evaluate the possible protective effect of EBCs against high light and UV-B radiation, we measured the growth of WT and *ebc-less* ice plants in response to combined elevated light intensity (450 μE) and ultraviolet B (UV-B) irradiation (32 $\mu W/cm^2$) (Figure S3A). WT and *ebc-less* plants grown under a light intensity of 180 μE for 9 weeks accumulated similar biomass (~0.17–0.23 g DW per plant) (Figure 2F). When subjected to UV-B and high-light conditions for 4 weeks after 5 weeks at 180 μE only, both genotypes exhibited increased growth, with WT plants producing more biomass (1.15 g DW per plant) than the *ebc-less* mutant (0.65 g DW per plant) (Figure 2F). The contents of chlorophyll *a* and *b* and total carotenoids increased to a greater extent in WT plants compared with *ebc-less* plants under exposure to UV-B and high-light conditions (Figures S3B–S3D).

Together, EBCs of the ice plant appear to serve protective functions in conditions of high light and UV-B radiation.

No effect of EBCs of quinoa in abiotic stress protection could be detected

In contrast to the ice plant mutant, the quinoa mutant *ebcf* grew to the same extent as the WT under UV-B and high-light conditions and did not show any differences in chlorophyll content or chlorophyll fluorescence parameters (Figures S4A–S4C). We exposed WT quinoa plants (cultivar 5206) and the *ebcf* mutant to other abiotic stresses, such as wind, waterlogging, heavy metals, high and low temperature, and low humidity. We did not detect a difference in growth between the two genotypes under any tested condition (Figures S4D–S4I). We subjected corresponding WT, *ebcf*, and *rebc-2* plants to a constant wind applied at a 45° angle to their shoot apex, as this has been previously shown to result in aberrant shoot apices in *rebc-2*.[22] Not only did wind-exposed plants of all four genotypes show the same decrease in DW (Figure S4J), but as in WT plants, the shoot apices of *ebcf* and *rebc-2* also developed similarly to those of control plants under wind treatment (Figures S4K and S4L).

The combined results suggest that EBCs do not protect quinoa from any abiotic stress examined. Furthermore, we could not confirm that the development of an aberrant shoot apex phenotype previously described in *rebc-2* plants is caused by abiotic stress. Consequently, EBCs likely have other roles.

Loss of EBCs makes quinoa and ice plants highly prone to thrips infestation

The above results were based on plants grown under controlled conditions in growth chambers. However, when quinoa and ice plants were grown in the greenhouse, all *ebcf* and *ebc-less* plants showed stunted growth relative to their respective WT, in addition to having aberrant apical meristems (Figures 3A and S5A), a phenotype that was also observed in *rebc-2*.[22] We observed similar phenotypes when growth chambers suffered from natural thrips infestations, which morphological analysis suggested were due to the invasive generalist herbivore *Frankliniella occidentalis*. To assess thrips feeding preferences, we grew each WT and mutant plant (24 per treatment) in individual

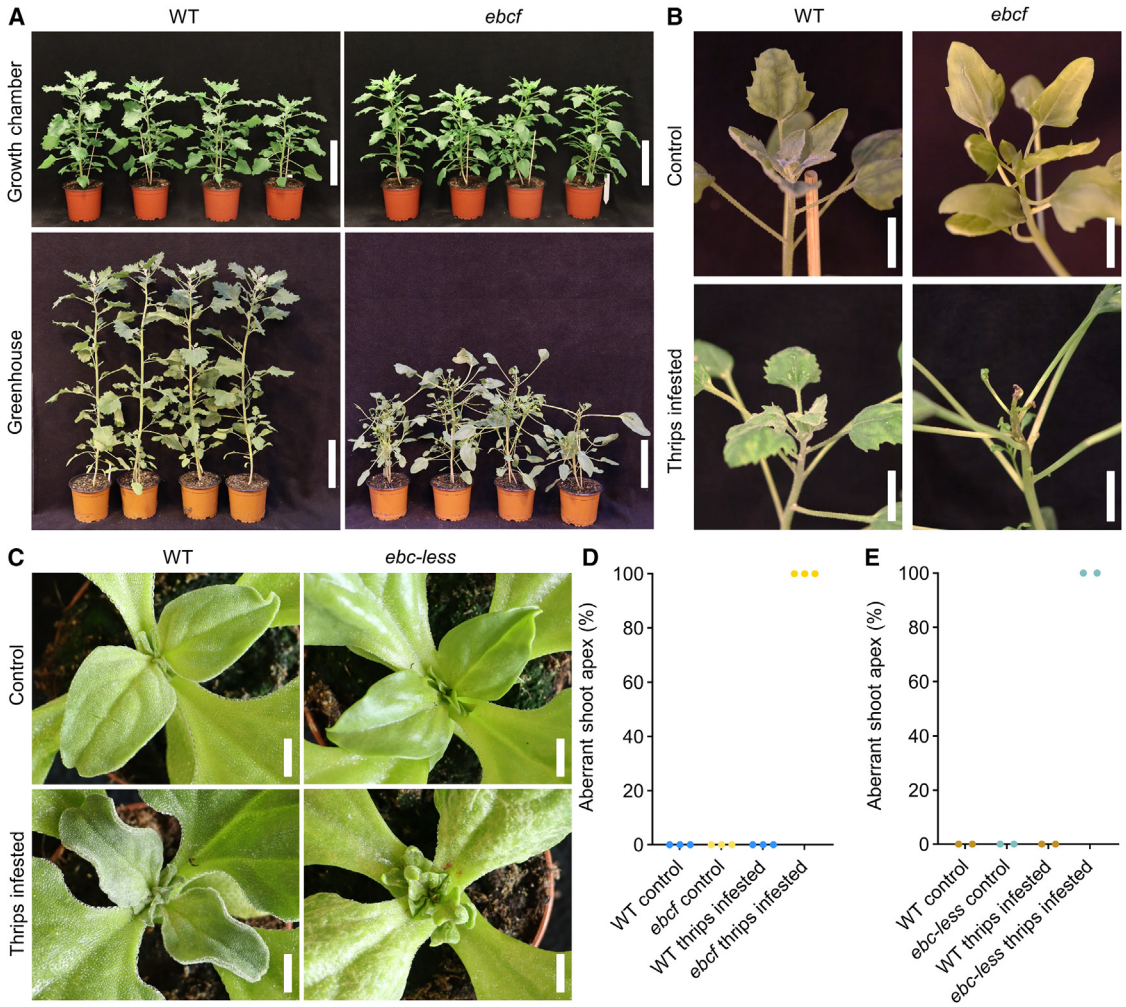

Figure 3. EBC-free mutants of both quinoa and ice plants suffer from thrips infestation

(A) Photographs of 7-week-old WT and *ebcf* plants grown in controlled growth chambers or in the greenhouse. Scale bars, 10 cm.

(B and C) Photographs of shoot apices of WT and *ebcf* quinoa plants (B) and WT and *ebc-less* ice plants (C) grown in thrips-impenetrable boxes either with no thrips added (control) or deliberately infested with thrips (*Frankliniella occidentalis*) (thrips infested) for 16 days. Scale bars, 5 cm.

(D) Relative appearances of the aberrant shoot apex phenotype of WT and *ebcf* quinoa plants grown in cages that either were not (control) or were infested (thrips infested) with thrips for 16 days. Filled circles represent individual values obtained from independent biological replicates, each including 24 plants.

(E) Relative appearances of the aberrant shoot apex phenotype of WT and *ebc-less* ice plants that either were not (control) or were infested (thrips infested) with thrips for 16 days. Filled circles represent individual values obtained from independent biological replicates, each including 24 plants.

This figure is related to Figures S4 and S5.

insect-free cages to shield the plants from herbivores. This produced healthy plants (*ebcf*, *rebc-2*, and *ebc-less* mutants and corresponding WT genotypes) with normal shoot apices (Figures 3B, 3C, and S5B). When we intentionally infested WT plants inside the cages with thrips, we observed thrips feeding only from fully expanded leaves, which have low EBC densities, and the plants developed normally (Figures 3B, 3C, and S5B). By contrast, the shoot apices of thrips-infested *ebcf*, *rebc-2*, and *ebc-less* plants died off (Figures 3B, 3C, and S5B). All the infested mutant plants with decreased EBC densities exhibited dead shoot apices within 16 days of infestation (Figures 3D, 3E, and S5C). Thus, mutant plants lacking EBCs are highly vulnerable to thrips infestations, which results in strongly impaired plant development.

EBCs form a physical barrier that protects the underlying tissue

To test how EBCs protect plants from feeding damage by small arthropod herbivores such as thrips, we first evaluated whether EBCs form a physical barrier that blocks access to the underlying plant tissue. We observed that the shoot apices of WT plants were completely covered with EBCs, whereas those of *ebcf* were naked (Figures 4A and 4B). We saw hardly any thrips on the shoot apices of WT plants, where EBCs occupied all the space in between leaves, and those thrips present were only able to occupy larger leaves that protruded from terminal buds (Figures 4A and S5D; Video S1). By contrast, the shoot apices of *ebcf* plants harbored more thrips and the thrips occupied the terminal bud (Figures 4B and S5D; Video S2). Furthermore,

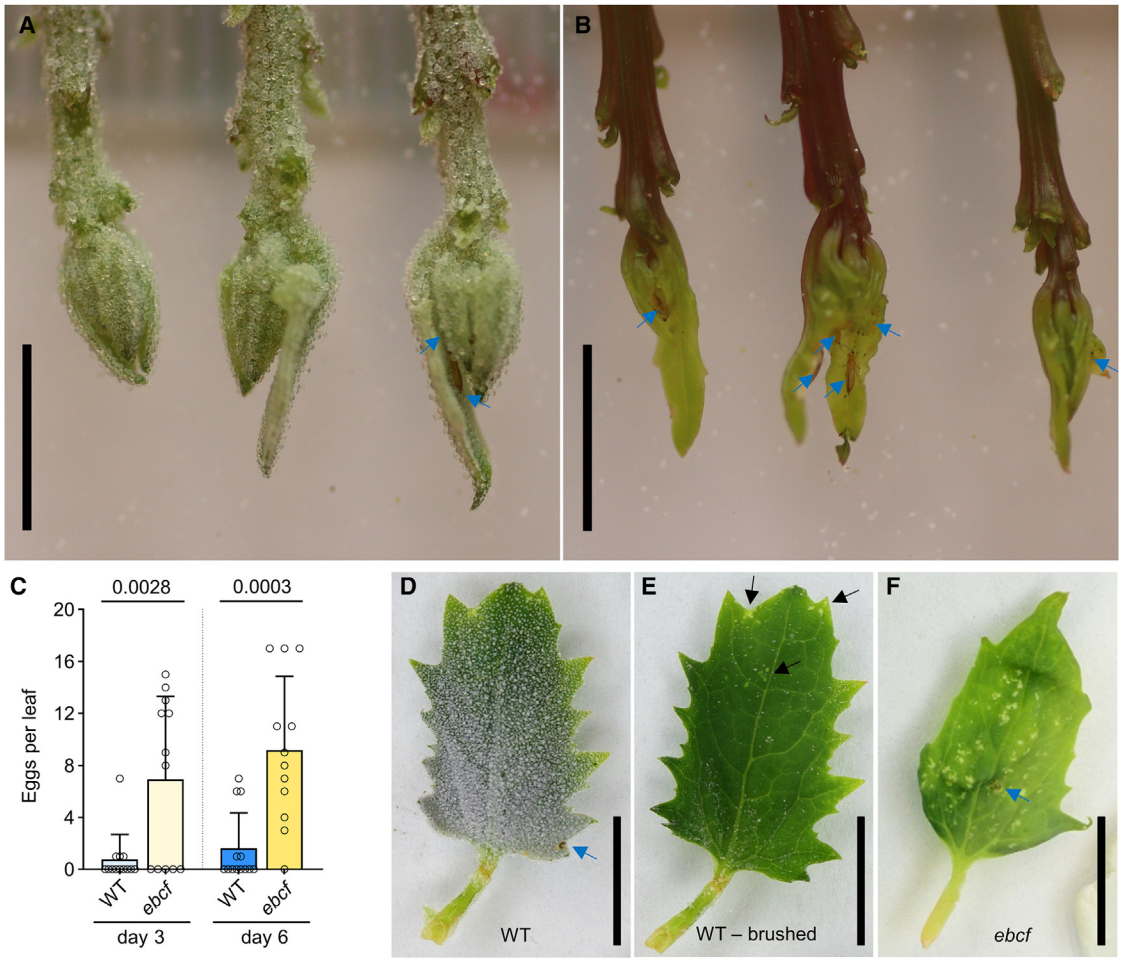

Figure 4. High densities of EBCs prevent access of small arthropod herbivores to the underlying plant tissue

(A and B) Photographs of the EBC-covered shoot apices of WT plants (A) and the EBC-free shoot apices of *ebcf* mutant plants (B) 1 h after being infested by thrips. Positions of thrips are indicated by blue arrows. Scale bars, 5 mm. See also Videos S1 and S2.

(C) Number of eggs laid by each female two-spotted spider mite (*Tetranychus urticae* London) that was presented with either a single emerging WT leaf completely covered with EBCs or with an *ebcf* leaf of the same age. Open circles represent individual values. Bar graphs represent mean ± SD; n = 13 leaves for WT and n = 12 leaves for *ebcf*. p values were obtained from an unpaired (two-tailed) t test.

(D) Photograph of a WT leaf that was subjected to an attack by a female two-spotted spider mite for 6 days. The position of a spider mite is indicated by a blue arrow. Scale bars, 5 mm.

(E) Photograph of the WT leaf shown in (D) after removal of EBCs by brushing. Feeding damage (yellow spots) is indicated by black arrows. Scale bars, 5 mm.

(F) Photograph of an *ebcf* leaf that was subjected to attack by a female two-spotted spider mite for 6 days. The position of the spider mite is indicated by a blue arrow. Scale bars, 5 mm.

thrips were able to move faster on leaves without EBCs (Videos S1 and S2).

The two-spotted spider mite (*Tetranychus urticae*) is a generalist arthropod herbivore that thrives on a diverse range of plant species and can rapidly adapt to new hosts.[23] It feeds by piercing mesophyll cells with a stylet of 100–150 μm in length and then consumes the cellular content protruding from the leaf surface.[24] As the diameter of EBCs equals that of the spider mite stylet, EBCs may provide an effective barrier to their penetration of the underlying leaf tissue. To test this possibility, we used young WT leaves completely covered with EBCs and *ebcf* leaves of identical size. We then allowed one female spider mite to move freely on the leaf. We counted the number of eggs laid by the spider mite on days 3 and 6 post infestation. We counted six times as many eggs per *ebcf* than per WT leaf (Figure 4C).

We inspected the behavior of spider mites on quinoa leaves and noticed that the high density of EBCs on the WT prevented access to the underlying leaf. WT leaves with high EBC density showed no feeding damage from spider mites, as the herbivores, when moving on the surface of these cells, appeared unable to reach the underlying cells. Also, EBCs themselves were not consumed and remained intact even after 6 days of feeding (Figure 4D). On WT leaves, only the outer edges, which have lower EBC densities, showed signs of feeding damage (Figure 4E). By contrast, evidence of feeding was detected throughout leaf laminas of *ebcf* quinoa leaves (Figure 4F).

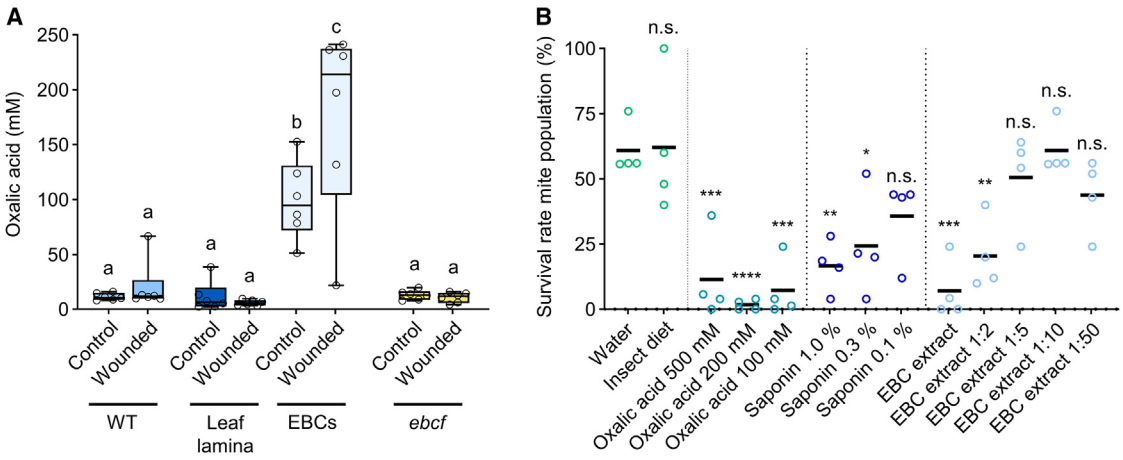

Figure 5. Oxalic acid is toxic to spider mites at concentrations detected in EBCs, as are EBC extracts

(A) Oxalic acid concentrations in whole leaves (13th leaf; WT) and the leaf lamina and EBC leaf fractions of WT plants with EBCs, as well as whole leaves of *ebcf* quinoa lacking EBCs, subjected to wounding (wounded) or left intact (control). On each plant, three leaves (8th, 9th, and 10th leaf of quinoa at the 16-leaf stage) were wounded three times with a pair of tweezers at 3-h intervals over the 24 h prior to sampling (with one-quarter of each leaf, beginning at the leaf tips, wounded at each 3-h interval). Open circles represent individual values. The boxes extend from the 25th to the 75th percentiles, with horizontal lines indicating the medians and whiskers ranging from minimum to maximum values; n = 6. p values were obtained from a two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Different letters indicate significant differences ($p < 0.05$).

(B) Survival rates of female two-spotted spider mites (*Tetranychus urticae* London) feeding on brilliant blue-dyed water, insect diet, EBC extracts, and solutions of oxalic acid and saponins for 48 h. Individual values are presented as open circles with mean as horizontal line, n = 4 independent experiments. p values were obtained from a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test, comparing each column with the water control and are indicated by asterisks ($p > 0.05$ n.s.; $p < 0.05$ *; $p < 0.005$ **; $p < 0.0005$ ***; $p < 0.00005$ ****).

This figure is related to Figure S6.

These results suggest that through a combination of their size and complete coverage of young plant tissues, EBCs prevent small arthropod herbivores such as thrips and spider mites from reaching the underlying leaf.

Oxalic acid concentration increases in EBCs upon wounding

We further tested the possibility that the EBC contents might offer a chemical barrier against spider mites, as the mites did not consume the EBCs and these cells contain high concentrations of potentially toxic metabolites such as oxalic acid and saponins.[18] We first measured the oxalic acid concentration in the EBCs of young quinoa leaves (leaves 13 and 14 at the 16-leaf stage). Whereas the concentration of oxalic acid in EBCs was 100 mM, in the underlying leaf cells it was below 10 mM (Figure 5A). To simulate feeding damage, we mechanically injured relatively mature leaves (leaves 8–10 at the 16-leaf stage) with a pair of tweezers three times at 3-h intervals. After 24 h, the oxalic acid concentration in EBCs from unwounded younger systemic leaves (numbers 13 and 14) had doubled (Figure 5A). Finally, transcript accumulation of putative marker genes for plant defense did not differ between the WT and *ebcf* upon wounding (Figures S6A–S6E).

EBC contents, including oxalic acid, are toxic to spider mites

Accordingly, we presented oxalic acid to spider mites at concentrations of 100, 200, and 500 mM and recorded their survival rates. Feeding on oxalic acid resulted in markedly reduced mite survival rates (≈5%) compared with feeding on regular tap water (62%) (Figure 5B). Saponin concentrations of 0.1% decreased survival rates only marginally, but at a saponin concentration of 0.3% and 1%, survival rates fell to 24% and 17%, respectively (Figure 5B). In addition, we presented EBC extracts to spider mites and recorded their survival rates. Survival rates were as low as 7% in mites presented with undiluted EBC extracts, similar to those with oxalic acid, but increased to ≈50% when the extracts were diluted 1:5, representing a survival rate close to that of the controls (Figure 5B). Together, these results suggest that EBCs accumulate oxalic acid to concentrations that are toxic to spider mites.

EBCs also deter a larger arthropod herbivore

To determine whether EBCs also deter larger herbivores, we tested the feeding preference of a leaf-chewing generalist herbivore. For this purpose, we allowed 1st-instar larvae from the beet armyworm (*Spodoptera exigua*) to feed from either WT or *ebcf* quinoa. The larvae grew bigger when feeding on the mutant and preferred young over older leaves (Figure 6). On average, *S. exigua* larvae weighed 11.4 mg when fed on WT plants but were more than twice as heavy when fed on *ebcf* (26.9 mg) (Figures 6A and 6B). Similar results were obtained when the larvae fed on *rebc-2*: the larvae weighed 4.8 mg on the corresponding WT and 8 mg on *rebc-2* after 8 days of feeding (Figures S7A–S7C). As EBC numbers are predetermined, densities are highest on emerging leaves and decrease during leaf expansion. In quinoa WTs, feeding damage was typically first seen on leaf 5 or 6 (counting from the first fully emerged leaf), while damage became visible as early as on leaf 2 with *ebcf* and *rebc-2* (Figures 6C–6E and S7D). We also performed a choice test in which 7- to 10-day-old 2nd-instar larvae were presented with intact WT leaves, WT leaves whose EBCs had been

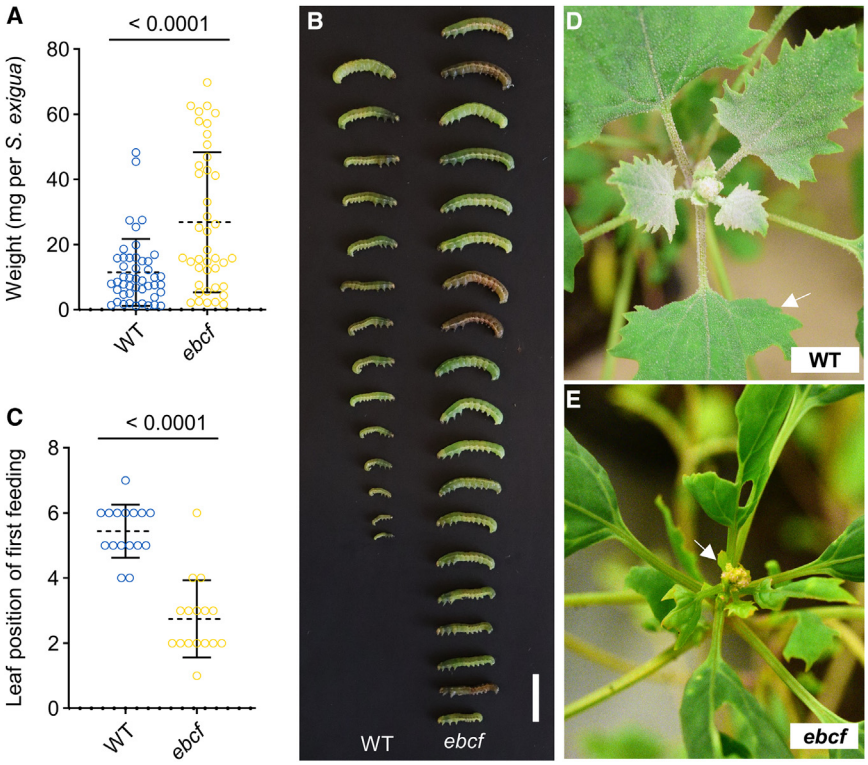

Figure 6. EBCs protect leaves from herbivory by *Spodoptera exigua*

(A) Larval weight of *S. exigua* after feeding on WT or *ebcf* plants for 9 days. Individual values are presented as open circles with mean ± SD, n = 46 and 41 larvae on WT and *ebcf* plants, respectively. p value was obtained through an unpaired (two-tailed) t test.

(B) Photograph of *S. exigua* larvae after 9 days of feeding on quinoa plants of each genotype. Scale bars, 1 cm.

(C) Position of youngest leaf with visible feeding damage (leaves counted from young—first leaf protruding from the terminal bud—to old). Individual values are presented as open circles with mean ± SD, n = 16 plants. p value was obtained through an unpaired (two-tailed) t test.

(D and E) Photographs of WT (D) and *ebcf* (E) plants after 9 days of being fed on by *S. exigua* larvae. Youngest leaf with visible feeding damage is indicated with an arrow in each case.

This figure is related to Figure S7.

removed by brushing, or *ebcf* leaves. The larvae preferred leaves without EBCs (Figures S7E–S7G). We observed no feeding preference when using old leaves with deflated EBCs in the WT (Figure S7H). We obtained similar results with ice plant leaves: leaves from the *ebc-less* mutant were chosen by 46.7% of the larvae, while the WT leaves were only chosen by 8.9% of the larvae (Figure S7I). Thus, EBCs of both quinoa and ice plant prevent not only small herbivores but also larger, generalist herbivores from feeding on young, developing leaves with high EBC densities.

EBCs prevent *Pseudomonas syringae* pv. *tomato* DC300 infection

We sprayed WT and *ebcf* quinoa plants with *Pseudomonas syringae* pv. *Tomato* (*Pst*) DC300 to test the effect of EBCs on leaf invasion by a pathogenic bacterium. Old leaves of the WT with few and deflated EBCs and *ebcf* leaves of the same age showed no difference in infection level (Figure 7). Although young leaves were infected to a lesser degree, *ebcf* leaves showed over 10-fold more colony-forming units/cm^2 than those of the WT (Figure 7). We conclude that the EBCs of quinoa form a physical barrier that prevents invasion by *P. syringae*, possibly by partly covering stomatal pores that are entry sites for this pathogen.

DISCUSSION

EBCs protect against biotic stresses

Here, we show that the primary function of EBCs of the tested species is to protect plants from biotic stress, such as herbivores and a pathogen, and not from various abiotic stresses as previously suggested.[9–11]

Since the cultivation of quinoa is extending throughout the world, the species faces extensive pressure from herbivores not occurring in their native range that cause significant damage.[7] However, with one exception,[19] little is known about how quinoa withstands the attacks of arthropod herbivores. In the above-mentioned work, it was suggested that EBCs prevent herbivory by insect pests in different chenopods, including quinoa. It was furthermore speculated that the EBCs must contain chemical compounds deterrent to herbivores.[19] Recent analyses of EBC content revealed high concentrations of oxalic acid, saponins, and betalains in these cells, suggesting that these compounds could function in defense against herbivory.[18] Thus, using the *ebc-less*, *rebc-2*, and *ebcf* mutants, we present support for the hypothesis that EBCs provide a mechanism for herbivory resistance in both quinoa and ice plant. EBCs are predominantly present on the abaxial leaf surface, where herbivores like caterpillars dwell.[25] Oxalic acid, saponins, and possibly other compounds present in these structures are toxic to many animals and thus form a chemical barrier that deters many generalist arthropod herbivores. Due to their large size and extensive coverage, especially on young leaves, EBCs create an additional physical barrier against small arthropod herbivores. Additionally, accumulating toxic compounds on the leaf surface—within EBCs rather than the underlying leaf cells—may offer extra advantages to the plant. These compounds are situated at the initial point of contact between the plant and herbivore and prevent autotoxic effects within the leaf.[26] This suggests that EBCs function as both physical and chemical barriers to generalist arthropod herbivore attack.

We further describe a novel function of EBCs. As EBCs cover leaves, they not only form a physical barrier to small arthropod herbivores but also partly cover stomatal pores, which are the entry points for many phytopathogens. The high density of EBCs prevents the entry of pathogenic bacteria (*Pst* DC3000) into the leaf and thereby protects plants from infections. We

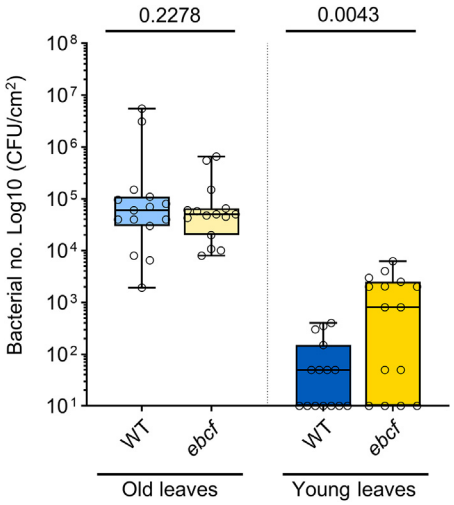

Figure 7. EBCs protect against infection by a bacterial pathogen

Quantification of *Pseudomonas syringae* pv. *tomato* DC300 bacterial populations in 5-week-old WT and *ebcf* plants 4 days post infection. Individual values are presented as open circles. The boxes extend from the 25th to the 75th percentiles, with horizontal lines indicating the medians and whiskers ranging from minimum to maximum values; n = 15 leaf disks from 5 plants. p values were obtained through an unpaired (two-tailed) t test.

hypothesize that this physical barrier is relevant not only to pathogenic bacteria but also to other phytopathogens such as downy mildew, which severely limits quinoa yields.[27]

Quinoa and ice plant EBCs play a limited role in abiotic stress tolerance

The idea that ice plant and quinoa EBCs serve as "salt dumps" stemmed from findings in certain *Atriplex* species that accumulate Na^+ in their EBCs.[28,29] Although K^+ concentrations decrease in EBCs upon salt treatment, Na^+ is actively loaded into these cells against a concentration gradient due to the overexpression of Na^+-specific transporters in EBCs of *Atriplex*.[30–32] In contrast to the situation in *Atriplex* species, here we confirm that ice plant EBCs contain Na^+ and Cl^- at concentrations comparable to those of the underlying cells, and consequently do not serve to concentrate salt, and that the EBC fraction does not increase upon salt treatment.[15,16] Similar observations were made in quinoa, where these cells accumulate Na^+ to the same or lesser extent than the underlying leaf.[17,18,33–35] Furthermore, and again contrary to findings in *Atriplex* species, transcriptomic analysis failed to provide evidence of active Na^+ loading into the EBCs of quinoa, as genes associated with Na^+ transport were not upregulated but those associated with K^+ transport were.[36,37] Indeed, an ice plant mutant lacking EBCs showed the same phenotypes as the WT under different salt and water stress conditions, providing genetic evidence that EBCs do not play a significant role in the salt and drought tolerance of this species. We already demonstrated that the *ebcf* quinoa mutant is as salt tolerant as its WT,[17] and here we confirmed this result with *rebc-2*, a quinoa mutant with greatly reduced EBC densities.

Furthermore, our results demonstrate that quinoa EBCs carry a fitness cost with respect to water stress tolerance. A likely explanation is that high accumulation of the osmolyte K^+ drives water into the EBCs. Recently, we showed that K^+ concentrations were lower in deflated EBCs than in turgid EBCs and that K^+ concentrations increased in EBCs when plants were osmotically stressed through salt treatments.[17] Here, we show that upon osmotic stress due to water withholding, K^+ concentrations also increase in EBCs to retain water and thereby keep the cells turgid. Only when K^+ concentrations also decreased did EBCs start to deflate, indicating that K^+ might be the major driver of water into EBCs to maintain the turgidity of these cells, even under conditions of osmotic stress and thus at the cost of water deficit tolerance. Furthermore, the accumulation of K^+ and subsequently water in EBCs reduces the succulence of the underlying leaf tissue, which decreases the overall drought tolerance of quinoa. Therefore, instead of EBCs being water reservoirs, as speculated,[6,12,13] the opposite seems to be true, as they seem to be osmotic sinks. Our results indicate that EBCs do not increase but rather decrease the plant's ability to withstand salt or drought stress, indicating that their utility lies in their herbivore defense properties.

We propose that salt and drought tolerance in quinoa and ice plant occur by alternative mechanisms: in quinoa, presumably via relocation of salt from young leaves to old leaves that subsequently wither and die[17]; and in ice plants, stress tolerance is enhanced via the accumulation of compatible solutes, as has been suggested.[38,39]

We observed a strong increase in growth in WT ice plants exposed to UV-B and high light intensities and a less pronounced growth promotion in the *ebc-less* mutant. However, we did not observe a similar protective effect of EBCs in quinoa. Further, as EBCs are more numerous on the abaxial surface of leaves in both ice plants and quinoa,[16,40] it is unlikely that they function solely as a protective barrier against UV stress. Nonetheless, the protective effects of EBCs in ice plants against high light and UV-B radiation merit further consideration. At high light intensities, EBCs of WT ice plants accumulate betacyanins and flavonoids, which have antioxidative properties.[21,41] Similarly, quinoa accumulates betacyanins such as betanin and amaranthin in EBCs, which may contribute to the pink/red coloration of these cells.[18] The intensity of EBC coloration is highly dependent on the cultivar. In this study, we used the quinoa cultivar 5206, which possesses relatively transparent EBCs. It is possible that EBCs provide protection in other cultivars that accumulate higher concentrations of betacyanins in EBCs. However, as betalains have been shown to deter herbivores,[42] high accumulation in EBCs could also add to the chemical barrier against arthropod herbivores. Furthermore, transcriptome and proteome analyses of salt-stressed ice plants revealed a marked increase in the abundance of transcripts and proteins associated with UV-B light protection.[43–45]

Our work demonstrates that the presence of EBCs reduces quinoa's ability to withstand water scarcity and enhances its resistance to arthropod herbivores and phytopathogens. Therefore, it would be of great interest to examine whether the varying fraction/density of EBCs among different quinoa cultivars correlates with their ability to withstand these stresses. If such a correlation exists, selecting quinoa with varying densities of EBCs based on the specific growth conditions could hold significant potential. For instance, cultivars with low EBC densities could be cultivated in areas with low herbivore pressure but regular

drought periods, while cultivars with high EBC densities could be selected for areas with opposite conditions. Such selection could minimize yield losses caused by abiotic and biotic stresses, consequently reducing the need for irrigation and pesticides and enhancing the sustainability of agricultural practices. Selecting cultivars with favorable chemical composition in EBCs could further add to this. Overall, with respect to the function of EBCs, this study has shifted the focus from abiotic to biotic stress resistance. This refocusing can aid the exploration of other mechanisms essential for abiotic stress tolerance while simultaneously opening numerous research avenues in the field of biotic stress resistance in quinoa.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.09.063.

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AUTHOR CONTRIBUTIONS

M.W.M. and M.P. conceived the study. M.W.M. and M.P. designed the experiments. M.W.M., X.Y., A.K.B., L.D., and C.C. conducted the experiments. M.W.M., M.R.K., and M.P. analyzed the data. M.W.M. wrote the first draft of the manuscript. T.I., M.M., J.C.C., and M.R.K. contributed biological material. All authors participated in writing and editing the manuscript. M.P. acquired funding and supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Pseudomonas syringae pv. *tomato* DC3000	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Gibco™ IPL-41 Insect Medium (1X)	Thermo Fisher Scientific	11405081
Oxalic acid	Sigma-Aldrich	194131
Saponin	Sigma-Aldrich	47036
Critical commercial assays		
Spectrum Plant Total RNA Kit	Sigma-Aldrich	STRN50-1KT
RNase-Free DNase Set	Qiagen	79256
QuantiTect® Reverse Transcription Kit	Qiagen	205313
HOT FIREPol(r) EvaGreen(r) qPCR Mix Plus (ROX)	Solis Biodyne	08-24-00020
Deposited data		
Main Figure Raw data	Mendeley	https://doi.org/10.17632/7rtrpcgpmy.1
Experimental models: Organisms		
Chenopodium quinoa cv.Titicaca	Moog et al.[17]	N/A
Ebcf	Moog et al.[17]	N/A
Chenopodium quinoa cv. CQ127	Imamura et al.[22]	N/A
rebc-2	Imamura et al.[22]	N/A
Mesembryanthemum crystallinum	Agarie et al.[16]	N/A
ebc-less	Agarie et al.[16]	N/A
Phaseolus vulgaris cv. Speedy	Widely distributed	N/A
Frankliniella occidentalis	This paper	N/A
Tetranychus urticae London	Merijn R. Kant Evolutionary and Population Biology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Netherlands	N/A
Spodoptera exigua	Entocare CV, Netherlands	N/A
Software and algorithms		
ImagingWinGigE v.2.47+	Walz	N/A
Mass Hunter Workstation Quantitation Analysis for QQQ, Version 10.1	Agilent Technologies	N/A
Excel 2016	Microsoft	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
GraphPad Prism, version 9	GraphPad Software	N/A
ImageJ	ImageJ software	N/A
Oligonucleotides		
Primers used for RT-qPCR, see Table S4	This paper	N/A

RESOURCE AVAILABILITY

Lead contact

Lead contact: Michael Palmgren; Email: palmgren@plen.ku.dk; Tel.: +45 2398 8444.

Materials availability

Materials and relevant information used in this study can be obtained from the lead contact, upon request.

Data and code availability

Raw data of figures were deposited on Mendeley at https://doi.org/10.17632/7rtrpcgpmy.1

This paper does not report original code.

Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant materials and growth conditions

In this study, wild-type (WT) and EBC-free (*ebcf*) *Chenopodium quinoa* Willd. cultivar 5206 (Titicaca) plants (described by Moog et al.[17]) and WT and mutant plants with reduced EBCs (*rebc-2*) of cultivar CQ127 (obtained and described by Imamura et al.[22]) were used. If not stated otherwise, the WT of cv. 5206 and the corresponding *ebcf* mutant were used for experiments. The WT and the *ebc-less* mutant of *Mesembryanthemum crystallinum* (obtained and described by Agarie et al.[16]) were analysed. For quinoa, two seeds were sown per pot in standard potting soil (Pindstrup Substrate, Ryomgaard, Denmark) and thinned to one seedling per pot after 1 week. Ice plant seeds were sown in soil, and individual seedlings were transplanted to bigger individual pots containing the same type of soil 3 weeks after sowing. Plants were regularly watered with tap water. Unless otherwise stated, plants were grown in controlled-climate growth chambers under the following standard conditions: 16 h light/8 h dark, 22°C/18°C (day/night), 60% relative humidity (RH), and 200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR).

METHOD DETAILS

Abiotic stress treatments

Progressive water deficit

Quinoa. Plants were grown in 50-ml pots for 30 days under standard conditions. Three days before water withholding, EBCs were removed from one set of WT plants by brushing with a cosmetic brush as described by Kiani-Pouya et al.[12] Prior to water withholding, pots with plants were allowed to stand in water for 8 h until the soil was completely soaked. Any remaining free water was removed from the trays containing the pots, and the plants were left in the growth chamber without watering for 7 or 9 days for cv. 5206 plants and for 8 days for cv. CQ127 plants before being irrigated again. Four days after watering resumed, the recovered plants were counted. Experiments were repeated three times (two times for *rebc-2*), and similar results were obtained each time. Furthermore, WT cv. 5206 and *ebcf* plants were used for pigment content analysis and chlorophyll fluorescence parameters. Relative water content (RWC) was measured for entire WT and *ebcf* plants, as well as for an old leaf (3rd leaf at the 14-leaf stage) and the youngest fully expanded leaf (12th leaf at the 14-leaf stage), by weighing plant tissue before and after drying at 80°C for 48 h. EBC fraction was too low to be measured in old leaves, but young WT leaves were separated into two leaf fractions (leaf lamina and EBCs) by collecting EBCs through gently scraping the leaf surface with a spatula, leaving an EBC-free WT leaf; RWC was then measured for each leaf fraction. Experiments were performed twice, with similar results each time. After weighing, these samples were subjected to ion content analysis. Leaf succulence was measured by weighing 1-cm^2 leaf disks. Leaves of 4-week-old plants were excised, and photographs were taken to analyse the total leaf area per plant using ImageJ software.

Ice plant. Plants were grown in 50-ml pots filled with a standardized amount of soil until they reached 5 weeks of age. Each pot was allowed to stand in water for 8 h in trays until the soil was saturated, and the pots were transferred into a new tray without water and watering was stopped. Control plants were irrigated sufficiently throughout the duration of the experiment. When the plants reached maturity, the emerging seed capsules were counted.

Continuous water deficit

Quinoa. WT quinoa and *ebcf* mutant seeds were sown on vermiculite. Soil-filled 0.5-liter pots were soaked to a soil water-holding capacity (WHC) of 100% and sealed with cling film. One-week-old seedlings were transplanted into the pots through a small hole in the cling film. The plants were grown without watering until the pots reached WHC values of 70% (control) or 20% (water deficit) (±2.5%). Pots were weighed daily and watered as needed to maintain the desired WHC until the plants were 6 weeks old. Photographs were taken, and DW was measured as described above. The biomass fraction of EBCs was obtained by weighing young fully expanded leaves of WT plants, removing the EBCs by brushing the leaves, and re-weighing the leaves. The relative biomass of each leaf fraction was then calculated. The experiment was performed twice with similar results.

Ice plant. For the ice plant experiment, WT and *ebc-less* plants were grown by a method similar to that for quinoa, except that seedlings were transplanted 3 weeks after sowing. The pots were irrigated to WHC values of 60%, 50%, or 40%. Depending on the set, the plants either had a constant WHC, had their WHC gradually reduced from 50% to 40%, or had it gradually reduced from 40% to 20% (±2.5%). After reaching the desired WHC values, the pots were watered daily to maintain the desired levels until the plants were 9 weeks old. The biomass of all plants was recorded.

Wind

WT seedlings of both cultivars and the corresponding mutants *ebcf* and *rebc-2* were prepared as described above for continuous water deficit in 0.3-liter pots sealed with cling film. Two-week-old seedlings were then subjected to a continuous wind stream from a mechanical fan that applied a constant airflow of 2.5 m/s at a 45° angle from above the shoot apices. Once per week, pots were watered to reach a soil WHC of 100%. Photographs were taken, and biomass recordings were taken after 4 weeks of wind treatment. The experiment was performed twice, with similar results.

UV/high light

Quinoa. WT (cv. 5206) and *ebcf* seeds were grown in 0.5-liter pots in a growth chamber under standard conditions for 2 weeks. The light intensity was then increased to 450 μmol m^{-2} s^{-1} PAR, and additional UV-B light was provided at an intensity of 32 μW/cm^2 (Reptile Desert Terrarium Bulb UVB 150, Exo Terra, Germany). Control plants were grown in a separate growth chamber with standard conditions. Plant biomass was recorded after an additional 5 weeks of growth. Leaf disks were excised from young leaves and their pigment contents were analysed. The experiment was repeated twice, and similar results were obtained. A second set of plants was grown for 5 weeks under standard conditions before being subjected to UV-B and high-light treatments. Chlorophyll fluorescence parameters were measured right before UV-B and high-light treatment was started and after 4, 9, and 24 h.

Ice plant. Ice plants were subjected to the same UV-B and high-light conditions described above starting on week 5 for 4 weeks before leaf disks were excised from a young fully developed leaf for quantification of chlorophyll and carotenoid contents. In addition, plant growth was determined by measuring DW as described above.

Salinity

Quinoa. WT (cv. CQ127) and the *rebc-2* mutant were grown under standard conditions in 0.5-liter pots for 2 weeks. Fourteen days after sowing, salt treatment was initiated via irrigation with 100 mM NaCl. NaCl concentration was doubled every second day until 400 mM NaCl was reached on day 5. Then biomass was recorded at week 6.

Ice plant. To test the growth response of *ebc-less* mutant plants to salinity, mutant and WT ice plants were grown in a controlled-climate growth chamber for 5 weeks. Salt treatments started at 35 days after sowing (DAS), with NaCl concentrations gradually increasing from 25% (37 DAS) to 50% (39 DAS) and finally to 100% of the final salt concentrations of 100, 400, and 800 mM NaCl, respectively, at 41 DAS. Control plants were grown without additional NaCl. Five weeks after the start of salt treatments, photographs were taken and biomass was recorded. Ionic content was measured in whole plants and in individual young (1^{st} leaf pair) and intermediate leaves (2^{nd} leaf pair). Whole dried plants were ground, and 20–100 mg of ground plant material was transferred into a 15-ml plastic tube for analysis. To measure ion contents in EBCs and the underlying leaf, the sap of EBCs was collected with a small glass capillary from a leaf patch of around 1 cm^2 taken from young and intermediate-aged leaves. The underlying leaf patch from which the EBC sap was removed was cut out and analysed as a second sample. A third patch was excised from a leaf with intact EBCs at an equivalent position. In addition, a leaf patch was excised from a mutant leaf for ion content analysis. Experiments involving growth of the WT and the *ebc-less* mutant in hydroponic culture were performed in a semi-controlled glasshouse in Copenhagen, Denmark, with the following conditions: 16 h light/8 h dark, 19°C/15°C (day/night); 65% RH; and 350 μmol m^{-2} s^{-1} PAR. For this purpose, seeds of the WT and the *ebc-less* mutant were sown on vermiculite and transplanted into five different 10-liter containers containing distilled water supplemented with 5 ml of a nutrient solution (Table S2) after 3 weeks. One week after transfer, KNO_3, NaCl, $NaNO_3$, and KCl were added to the hydroponic solution individually to each container to a final concentration of 100 mM. The concentration was doubled every second day until reaching 400 mM on day 5. As a control, water was added to one container. Four weeks after the first salt application, plants were harvested and their DW recorded as described above.

Waterlogging

For the waterlogging treatment, plant pots containing 2-week-old quinoa seedlings grown at standard conditions were placed inside 1-liter plastic pots, which were then filled with tap water to the level of the soil surface. Potted seedlings were kept at this water level for 4 weeks. Plants were then weighed after 6 weeks.

Heavy metals

Growth conditions were similar to those used for salinity treatments, but with the addition of 25 μM $ZnSO_4$ after 2 weeks, with the concentration doubled every other day to reach a final concentration of 100 μM $ZnSO_4$ at 18 DAS. $ZnSO_4$ irrigation continued for another 17 days, and then plant biomass was recorded.

High temperature

WT and *ebcf* plants were grown in a growth chamber for 6 weeks under the following conditions: 16 h light/8 h dark, 32°C/23°C (day/night), 80% RH, and 200 μmol m^{-2} s^{-1} PAR. The biomass of 6-week-old plants was measured.

Low temperature

Seedlings were grown under standard conditions for 2 weeks. The temperature was then lowered to 10°C during the day and 5°C during the night for 6 weeks, and the biomass was recorded.

Low humidity

Plants were grown for 6 weeks under standard conditions but with the relative humidity adjusted from 60% to 30%. After 6 weeks of growth, plant biomass was measured.

EBCs as transpirational barrier

WT plants were grown alongside *ebcf* plants for 5 weeks under standard conditions on soil as described above. EBCs were removed from one side of a WT leaf on the adaxial and abaxial sides by brushing. Three days later, the transpiration rate of each half of the leaf (brushed and unbrushed) was measured separately using a portable gas-exchange fluorescence system, as well as that of similarly sized leaves from *ebcf*. The experiment was repeated twice, and similar results were obtained.

Disease test with *Pseudomonas syringae* pv. *tomato* DC3000

A virulent culture of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 was grown in LB medium containing 25 μg ml^{-1} rifampicin at 28°C in a shaking incubator to late log phase. *Pst* was harvested by centrifugation at 2,348 rcf (relative centrifugal force) for 7 min at room temperature and then resuspended in 10 mM $MgCl_2$ and 0.01% (v/v) Silwet 77. The cell suspension was diluted to an OD_{600} of 2.23 with 10 mM $MgCl_2$ and then sprayed, until all leaves were dripping wet, onto 5-week-old WT and *ebcf* quinoa plants that had been grown under standard conditions in 0.3-liter pots and kept in the dark for 20 h prior to spraying. Plants were then returned to the growth chamber and covered with a translucent plastic hood to ensure high humidity. After 24 h, the hood was removed. Young leaves that were newly emerging on the day of infection, as well as old leaves (3^{rd} leaf), were chosen for analysis. Four days after infection, three leaf disks from each young and old leaf were ground in 200 μl 10 mM $MgCl_2$ and then 800 μl 10 mM $MgCl_2$ was added. A series of 10-fold dilutions were made, and 20-μl drops from each dilution were spotted onto LB + rifampicin plates (25 μg ml^{-1}). After 3 days of incubation at 30°C, the number of colonies was counted and normalised to the leaf area of the leaf disks. The experiment was performed twice, and similar results were obtained.

Mechanical wounding

Quinoa WT (cv. 5206) and *ebcf* plants were grown under standard conditions in 0.3-liter pots for 4 weeks until they reached the 16-leaf stage. Wounding was then applied to the 8^{th}, 9^{th}, and 10^{th} leaves in 3-h intervals. Wounds were made with a pair of tweezers by squeezing 25% of the leaf during each interval, beginning from the tip of the leaf (Figures S6F and S6G), until 75% of the leaf was covered with wounds. Twenty-four hours after the first wound, wounded leaves were harvested for RT-qPCR analysis, while intact 13^{th} leaves were collected for liquid chromatography–tandem mass spectrometry (LC-MS/MS). For LC-MS/MS, each 13^{th} leaf was divided into two halves along the main vein. One half was left untouched, while EBCs were collected from the other half by gently scraping the leaf surface with a spatula, resulting in an EBC sample and a sample of leaf lacking EBCs. Controls were harvested from leaves of unwounded plants. All samples were immediately snap frozen in liquid nitrogen until further analysis.

Insect bioassays

Western flower thrips (Frankliniella occidentalis)

WT quinoa plants (cv. CQ127 and 5206) and the corresponding mutants *rebc-2* and *ebcf*, as well as WT ice plant and the *ebc-less* mutant, were grown in 0.05-liter pots under standard conditions for 3 weeks. 24 plants of each genotype were then placed in individual Plexiglas boxes with air inlets covered with a thrips-impermeable mesh and placed in a greenhouse. Thrips, which were identified by eye to be *F. occidentalis*, were shaken off an infested quinoa plant into petri dishes. A petri dish containing around 100 thrips was then placed into each Plexiglas box (thrips-infested condition), while another box was kept closed to avoid any contamination (uninfested control condition) (Figure S5E). Infestation rates were obtained by checking all plants for the phenotype observed previously for greenhouse-grown plants and also found in *rebc-2* plants (as described by Imamura et al.[22]), which consists of damaged shoot apices. After 16 days, the rate of appearance of this phenotype was recorded. This experiment was repeated twice for *ebcf* and once for *rebc-2* and *ebc-less*. Then, the last ~1.5 cm of the shoot apex was excised from the plant, and the larger attached leaves were removed and immediately placed into a petri dish that was then sealed with Parafilm. Using a stereomicroscope (EZ4; Leica), each petri dish was evaluated for the presence of thrips and then opened. Remaining leaves were removed individually (Figure S5F), and the number of thrips was recorded. In addition, ~20 thrips were placed in a petri dish and the last 5 cm of 7 WT and *ebcf* shoots from which all leaves except the terminal bud had been removed were placed inside the petri dish with the thrips. Starting 30 min later, videos of the thrips moving on and inside the terminal buds were obtained to record the behaviour and the movement of the thrips over a period of 20 min.

Two-spotted spider mites (Tetranychus urticae)

A colony of the two-spotted spider mite strain *T. urticae* London was kept in controlled-climate chambers (25°C, 16-h light/8-h dark, 60% RH, and 300 μmol m^{-2} s^{-1}) on detached leaves of *Phaseolus vulgaris* cv. Speedy placed on wet cotton wool. To ensure that

spider mites of the same age were used, before each experiment female mites were collected from the original colony and placed on a new tray containing detached *P. vulgaris* leaves on wet cotton wool. Female spider mites were then removed after 3 days, leaving the eggs they had laid on the leaves. All experiments were conducted with 14-day-old female spider mites from these fresh colonies. Emerging leaves of the WT completely covered with EBCs and *ebcf* leaves were excised with a razor blade from 3-week-old plants, and their petioles were placed into 1% (w/v) agarose in small plastic cups at an angle of 45°. On each leaf, one female spider mite was placed next to the petiole, and the cups were then sealed with lids containing a nylon mesh window. Cups were returned to the growth chamber, and the number of eggs were counted on days 3 and 6. On day 6, leaves were removed from the cups and photographs were taken.

Artificial diet feeding of spider mites

WT plants (cv. 5206) were grown in 0.3-liter pots for 4 weeks under standard conditions. EBCs were collected from young leaves by scraping as described above and placed in a 1.5-ml tube containing five to seven 0.5-mm glass beads. EBCs were vortexed briefly and centrifuged at 12,000 rcf for 5 min at room temperature. The supernatant was collected, and four dilutions were made in tap water (1:2, 1:5, 1:10, and 1:50, v/v). Brilliant blue FCF powder was added to the undiluted EBC extract and to each dilution to ~1% (w/v). Feeding arenas were prepared by placing 2-cm^2 nylon mesh on an upside-down 60 x 15-mm petri dish modified as described by Ghazy et al.[46] At the centre of each nylon mesh, 35 μl of EBC sample was pipetted and covered with a piece of stretched Parafilm, being sure to avoid trapping air underneath, with the EBC sample being only on the nylon mesh. Strips of water-soaked tissue paper were placed around the covered nylon mesh to restrict the mites from moving off the feeding arena. Then, 25 14-day-old female two-spotted spider mites reared on detached common bean (*Phaseolus vulgaris* cv. Speedy) leaves were placed onto the arena. Spider mites trapped in the tissue paper were placed back onto the arena after 24 h. After 48 h, blue mites were scored for evidence of feeding, and the alive and dead mites were counted. Additional feeding arenas with different concentrations of oxalic acid (CAS No. 144-62-7) (0.5, 0.2, and 0.1 M) and saponin (CAS No. 8047-15-2) (0.1, 0.3, and 1.0%) were prepared as above. As controls, arenas were prepared with Gibco™ IPL-41 Insect Medium (1X) (Thermo Fisher Scientific; 11405081) artificial insect diet and with regular tap water. The experiment was repeated three times, and similar results were obtained each time.

*Beet armyworm (*Spodoptera exigua*)*

Quinoa. S. exigua eggs were obtained from Entocare CV (Netherlands) and reared on an artificial diet (Table S3) until needed. **Non-choice tests.** Four 1st-instar *S. exigua* larvae were placed on individual 4-week-old quinoa plants of the two WT cultivars and *ebcf* and *rebc-2* mutants, in separate insect-free boxes (one box per genotype). Larvae were able to move freely within each box and from plant to plant. After 9 days, the mass of individual *S. exigua* larvae was determined by weighing, and photographs of plants and *S. exigua* larvae were taken. Beginning from the youngest leaf, the number of the first leaf showing visible feeding damage was recorded. Experiments were performed twice for cv. 5206, and similar results were obtained. **Choice tests.** Choice tests were performed with 7- to 10-day-old 2nd instar *S. exigua* larvae. Leaves from 4- to 5-week-old plants were excised with a razor blade and the petiole placed into 1.5-ml tubes filled with water and sealed with Parafilm. Young intact leaves from the WT, leaves from WT whose EBCs were removed by brushing with a cosmetic brush, and leaves from the *ebcf* mutant were used. In addition, intact old leaves from WT and *ebcf* plants were included. Two leaves from different combinations of genotypes were placed into a petri dish, and one 2nd-instar *S. exigua* larva was placed in between the two leaves and was able to move freely between them. After 20 h, feeding preference was recorded by visual observation. If no preference was noted, or only marginal leaf consumption was detected, "no preference" was recorded. Each experiment was repeated two or three times, and similar results were obtained.

Ice plant. **Choice tests** were performed on young leaves as described above, and feeding preferences were recorded after 48 h. Options for recording were either preference for WT over *ebc-less* leaf or vice versa; if no clear feeding preference was observable, "no preference" was recorded.

Gas-exchange measurements

A GFS-3000 portable gas-exchange fluorescence system (Walz, Effeltrich, Germany) with measuring gas set to resemble conditions in the growth chamber (flow rate: 900 μmol min^{-1}, H_2O: 12,425 ppm; CO_2, 400 ppm; temperature, 23°C) was used to evaluate transpiration rates. Leaves were individually clamped into a measuring head of 2.5 cm^2 and were illuminated with 200 μmol m^{-2} s^{-1} from the 3041-L LED light source (warm-white LEDs; Walz).

Pigment analysis

Total chlorophyll and carotenoid contents of a 0.5027-cm^2 leaf disk were acetone-extracted (80% [v/v] in water), and absorbance of the pigment solution was measured at 470, 646.8, and 663.2 nm with a spectrophotometer (Spectronic Genesys 10 Bio, Thermo Scientific, Waltham, MA USA). Pigment concentrations were calculated as described by Lichtenthaler and Buschmann[47] and normalised to the area of the analysed leaf disk.

Chlorophyll fluorescence parameters

Chlorophyll fluorescence from excised and dark-adapted leaves (placed in 1.5-ml tubes filled with tap water and covered with an aluminium foil–covered cardboard box for 15 min) was measured with an Imaging-Pam-Max/L (Walz, Effeltrich, Germany) and analysed with ImagingWinGigE v.2.47+ software (Walz). The effective quantum yield of photosystem II (PSII) was determined, which was defined as $F_v/F_m = F_m - F_0)/F_m$.

LC-MS/MS

Approximately 30–40 mg of leaf discs or scraped EBCs were collected and frozen in liquid nitrogen. The frozen samples were ground using a pestle, and compounds were extracted in 200 μl extraction buffer (1 μM Schaftoside in 85% [v/v] aqueous methanol) for 5 min on ice. The extracts were collected by centrifugation for 2 min at 21,300 rcf at room temperature and filtered through a membrane filter (0.22 μm, Merck Millipore). Samples were diluted 200-fold with deionized water and subjected to analysis by LC-MS/MS. Chromatography was performed on a 1290 Infinity II UHPLC system (Agilent Technologies). Oxalic acid was analysed using the following chromatography conditions. Separation was achieved on a Kinetex Biphenyl column (100 x 2.1 mm, 1.7 μm, 100 Å, Phenomenex, Torrance, CA, USA). Ammonium acetate (2 mM, pH 6.6) and methanol were employed as mobile phases A and B, respectively. The elution profiles were as follows: 0–1.0 min, 3% B; 1.0–2.5 min, 3–35% B; 2.5–3.0 min, 35–75% B; 3.0–3.1 min, 75–100% B; 3.1–4.1 min, 100% B; 4.1–4.2 min, 100–3% B; and 4.2–5.5 min, 3% B. The mobile-phase flow rate was 400 μl/min. The column temperature was maintained at 40°C. Injection volume was 1 μl. A liquid chromatograph was coupled to an Ultivo Triple Quadrupole mass spectrometer (Agilent Technologies) equipped with a Jetstream electrospray ion source (ESI) operated in combined positive- and negative-ion modes. The instrument parameters were optimized by infusion experiments with pure standards. The ion spray voltage was set to 4,000 and 3,000 V in positive- and negative-ion modes, respectively. Dry gas temperature was set to 325°C and dry gas flow to 13 liters/min. Sheath gas temperature was set to 265°C and sheath gas flow to 12 liters/min. Nebulising gas was set to 50 psi. Nitrogen was used as dry gas, nebulising gas and collision gas. Multiple reaction monitoring (MRM) was used to monitor analyte precursor ion → fragment ion transitions. MRM transitions were determined by direct infusion experiments of reference standards. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Ionization efficiency was checked by analysing dilution series, which were also used for quantification. m/z for the precursor ion is 89 (negative ion mode, [M-H]-) and transitions are 89-61 (quantifier) and 89-45 (qualifier). The Mass Hunter Workstation Quantitation Analysis for QQQ software (Version 10.1, Agilent Technologies) was used for data processing and analysis.

Ion analysis

Ion contents of dried plant material were extracted in 0.5 M HNO_3 as described by Munns et al.[48] Concentrations of Na^+ and K^+ were calculated using standards containing the analysed nutrient using a PFP7 Flame Photometer (Jenway, Stone, UK) and normalised to the dry weight of the analysed samples. Cl^- was determined using the Chloride Analyser 926 (Sherwood Scientific, UK).

RNA extraction, cDNA synthesis, and RT-qPCR analysis

Leaf samples were obtained as described in the "mechanical wounding" section. In brief, each treatment group consisted of 6 plants, with each plant having 3 leaves subjected to wounding. The leaves were removed at the base, excluding the petiole, and the three wounded leaves from each plant were combined. This process resulted in six distinct samples per treatment group. The individual leaf samples were immediately flash frozen in liquid nitrogen and stored at -80°C. Still frozen the leaves were ground to a powder, and total RNA was extracted from approximately 100 mg of this powdered tissue using the Spectrum Plant Total RNA Kit (cat. no. STRN50-1KT; Sigma). Genomic DNA was degraded with an on-column DNase I digestion step using an RNase-Free DNase Set (cat. no. 79256; Qiagen). The quality and concentration of the extracted RNA were assessed using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). First-strand cDNA synthesis was carried out using the QuantiTect® Reverse Transcription Kit (Qiagen, cat. no. 205313) using 1 μg DNAse-treated total RNA. RT-qPCR was performed on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA USA) using a HOT FIREPol(r) EvaGreen(r) qPCR Mix Plus (ROX) (cat. no.08-24-00020; Solis Biodyne). The PCR program of three segments was as follows: segment 1 was 2 min at 50°C, 15 min at 95°C; segment 2 was 40 cycles of 15 s at 95°C, 30 s at 60°C, and 60 s at 72°C; and segment 3 was 15 s at 95°C, 60 s at 60°C, and 30 s at 95°C. For each reaction, 5 ng of cDNA was used as the template in a final volume of 20 μl. Negative control samples lacking cDNA were included. Cq (CT) values were calculated according to the default settings of the ABI 7500 Real-Time PCR system using the comparative CT (ΔΔCT) method. *Elongation factor 1α* (*Ef1α*) was used as a reference gene, and primer sequences were obtained from Böhm et al.[36] Expression of the following putative defence genes were analysed: *EOT1*, *TPS5*, *PPOD*, *PR1α* and *PI-IIc* (primer sequences are provided in Table S4). To assess PCR specificity, PCR products were sequenced and subjected to melting curve analysis subsequent to each PCR program. Each sample was represented by one technical replicate on the qPCR plate, except for Ef1α, where each sample was represented by two technical replicates. Normalization was performed using the delta-Ct method as detailed by Alba et al.[49] The resulting values were normalized to the expression observed in control samples.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

All statistical analyses were performed using Excel 2016 (Microsoft, Redmond, WA, USA) and Prism 9 (GraphPad Software, San Diego, CA, USA), and graphs were plotted using Prism 9. Statistical differences were calculated by Student's *t*-test for pairwise comparisons. For multiple-group comparisons the mixed-effects model (REML) was used in cases with missing values, one- or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test (in case of no missing values), or by Dunnett's multiple comparisons test, when comparing multiple columns with a control column. Statistical tests were performed using Prism 9 using recommended settings. Further details on statistical analysis are presented in the figure legends.