Friend or Foe? Chloride Patterning in Halophytes

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Abstract

In this opinion article, we challenge the traditional view that breeding for reduced Cl^- uptake would benefit plant salinity tolerance. A negative correlation between shoot Cl^- concentration and plant biomass does not hold for halophytes, naturally salt tolerant species. We argue that under physiologically relevant conditions, Cl^- uptake requires plants to invest metabolic energy, and that the poor selectivity of Cl^--transporting proteins may explain the reported negative correlation between Cl^- accumulation and crop salinity tolerance. We propose a new paradigm: salinity tolerance could be achieved by improving the selectivity of some of the broadly-selective anion-transporting proteins (e.g. for NO_3^- > Cl^-), alongside tight control of Cl^- uptake, rather than targeting traits mediating its efflux from the root.

Chloride - A Toxin or a Beneficial Osmoticum?

The physiological and molecular mechanisms conferring plant salinity tolerance have been intensively studied over the last four decades. Most investigations have focused on Na^+, but the past few years have witnessed a 'renaissance period' for Cl^- research. This interest is mainly driven by the need to fully understand the role of Cl^- as a nutrient and its conflicting role in limiting plant growth in saline soils [1-5]. However, this current attention on the role of Cl^- in plant salinity responses has concentrated on non-halophytes. Is Cl^-, when present at high concentrations in cells, 'toxic' to all plants and via what mechanism(s) (see Outstanding Questions)? Is plant breeding for Cl^- **exclusion** (see Glossary) the best way to proceed? For Na^+, studies on halophytes have instigated a paradigm shift in the suggested approach to crop breeding for salt tolerance [6]. Should this be considered also for Cl^-? We argue here that **halophytes** require Cl^- as an **osmoticum** for growth and *invest* metabolic energy for its uptake, even under saline conditions. We then discuss the molecular identity and regulation of Cl^- **transport proteins** in halophytes as well as the implications of these findings for breeding salt tolerant plants.

Should Chloride be Excluded?

In non-halophytes, the current notion is that Cl^- exclusion from the shoot (either from the root epidermis or the xylem) is crucial for salt tolerance [1, 3, 7-12]. These arguments are supported by findings in some salt-sensitive species that high shoot Cl^- levels correlate with severe physiological dysfunctions that not only affect yield but also the quality and edibility of non-halophytic crops [4, 13, 14]. Nonetheless, tissue Cl^- concentrations in halophytes can exceed 500 mM [15, 16]. In some extreme dicotyledonous halophytes (including those that do not rely on salt glands or bladders for ion homeostasis), viable shoot tissues can contain more than 1.5 M Cl^- [17, 18]. Thus, the reported negative correlation between shoot Cl^- concentration and plant biomass in some salt-grown non-halophytes does not hold for halophytes (Fig 1). The physiological rationale behind the high shoot Cl^- concentrations in halophytes is, first, that accumulating Cl^- is energetically less expensive than excluding it, particularly because it minimises the need for biosynthesis of organic solutes to achieve full osmotic adjustment [19]. Second, Cl^- is well suited as a solute for stomatal opening as its transport across the guard cell membranes is cheaper than the biosynthesis of malate; in most plant species, malate is used for stomata opening [20]. Finally, several

dicotyledonous halophytes require Cl^- in the high mM range (200-500 mM) for optimal photosynthetic activity [21-25]. These points, combined with recent findings that Cl^- at mM levels is a beneficial for cell turgor and growth in at least some non-halophytes [2, 26], raise the question of whether breeding for Cl^- exclusion is the way to proceed. Breeding for either NaCl ion exclusion or **tissue tolerance** are two contrasting strategies that are currently hotly debated in the salinity research field [6, 19, 27-30]. Addressing this question requires a rigorous understanding of ion (Cl^- and Na^+) regulation in halophytes and comparative studies with species across the salt tolerance continuum. This will not only allow quantification of the importance and limitations of these two contrasting adaptive strategies across the salt tolerance continuum, but will also allow old paradigms to be revisited, opening new prospects for improving crop salinity tolerance.

Energetics of Chloride Transport

The cellular targets for Cl^- toxicity remain elusive, but high Cl^- concentrations have been found to interfere with root NO_3^- uptake [31], stomatal regulation and leaf photosynthetic capacity [11, 32]. Thus, using Cl^- for osmotic adjustment comes with the risk of overaccumulation and potential toxicity to cellular functions. In most plant cells, the large central vacuole serves as a storage reservoir, where during salt stress, cytotoxic Cl^- and Na^+ are sequestered away to maintain optimal physiological conditions in the cytosol. Nevertheless, although salt tolerance in halophytes is in part associated with efficient vacuolar sequestration of Cl^- (with evidence indicating stronger Cl^- selectivity and differential transport kinetics for some vacuolar Cl^- transporters in halophytes compared to non-halophytes [33, 34]), Cl^- regulation in halophytes must be underpinned by an ability to tightly control, in time, net Cl^- uptake at the root plasma membrane (PM) to match plant demand. Indeed, very small imbalances between the rate of Cl^- supply with respect to its accumulation and subsequent sequestration away from the cytosol in the vacuoles would have grave consequences given the relatively small cytosolic volumes. Supporting this hypothesis is the observation that, despite **hyperaccumulating** Cl^- at low NaCl concentrations (well above the levels required to match soil osmotic potentials, for example at 10-100 mM NaCl [15, 35] (Figure 1)), halophytes still maintain relatively stable internal shoot Cl^- concentrations when exposed to a wide salinity range [15, 35]. By contrast shoot Cl^- concentrations show greater relative increases with increasing salinities in

non-halophytes [35]. How do halophytes maintain relatively stable shoot Cl^- concentrations?

Being a negatively charged ion, Cl^- uptake constitutes thermodynamically **active ion transport** under the Cl^- concentrations found in most non-saline agricultural soils (below 40 mM NaCl [11, 36]). Indeed at low salinities (10-60 mM NaCl), Cl^- uptake is proposed to be mediated by $Cl^-/2H^+$ **symporters** using energy from the H^+ **electrochemical difference** across the PM [8, 31, 36, 37]. Can Cl^- uptake become passive (channel-mediated) under saline conditions (100 mM NaCl or more)? Although passive Cl^- uptake has been proposed [38], this scenario is unlikely for the majority of Cl^- concentrations in field conditions, including most of the high soil Cl^- levels faced by salt-tolerant halophytes. Concentration gradients across a membrane (i.e., cytosolic and extracellular Cl^- concentrations) and the cell membrane potential will determine the requirement active ion transport or passive influx/efflux [8]. Thus, based on the scarce available data, our calculations using the Nernst equation indicate that Cl^- influx into halophyte roots is likely to be an active process up to 600 mM external NaCl (Figure 2; calculations are given in the supplemental information online). Indeed, the prerequisite for passive influx of Cl^- is that PM potentials need to be less negative than the Cl^- **equilibrium potential** (E_{Cl}). Hence, passive uptake would only occur in response to very rapid increases in soil salinity, which result in a rapid and transient PM depolarisation (as demonstrated in both non-halophytes and halophytes, where root PM potentials increase from -140 and -130 mV to between -40 and -50 mV, respectively [39]) while cytosolic Cl^- ($[Cl^-]_{cyt}$) remains low (Figure 2; also [8]). This rapid passive Cl^- influx would stop PM depolarisation and increase $[Cl^-]_{cyt}$. This, combined with the partial/full recovery of root PM potential, that in halophytes occurs within hours after the salt treatments [40, 41] while in non-halophytes salt-induced depolarisations remain stable over days [42], would quickly reinstate the conditions where active Cl^- uptake is required. Passive Cl^- influx could therefore operate for a very short period (minutes to a few hours) and is unlikely to have much relevance to agricultural field conditions where NaCl increases are gradual [43]. Therefore, for the majority of physiological situations, Cl^- uptake is thermodynamically active, and plants need to invest metabolic energy to acquire Cl^-, even under conditions of high (up to 600 mM Cl^-) soil salinity.

Why do some plants putatively 'waste energy' taking up Cl^- only to later efflux it (e.g. [44]), maintaining a net root Cl^- 'exclusion'? Indeed, some reports indicate that passive Cl^- efflux via anion channels in non-halophyte roots could represent up to 90% of the total root-acquired Cl^- [44]. While the reason for such 'futile cycling' (*cf.* [45]) remains to be answered, the finding of large amounts of Cl^- efflux from roots of non-halophytes has led to the hypothesis that root Cl^- efflux is crucial for Cl^- exclusion under saline conditions, limiting Cl^- accumulation and thus enhancing salt tolerance in plants [7, 12, 44]. Although definite conclusions cannot be drawn from the limited data available, halophytes could provide information on alternative mechanisms to regulate Cl^- transport at the root PM. Because halophytes have evolved to thrive in NaCl-rich soils, it is logical to expect that they have optimised ion regulatory processes and energy costs. Nonetheless, the seemingly 'futile cycling' in Cl^- transport at the root PM would represent a large reduction in overall energy efficiency (as proposed for Na^+, [45]), a situation that appears to be counterproductive for growth in saline habitats where plants would be expected to limit their costs. We therefore propose that in halophytes, under *quasi* steady-state conditions, the ability to control Cl^- influx (as opposed to relying on high rates of efflux) is the key feature of their capacity to tightly control net Cl^- uptake at the root PM. Considering that ion transport processes are a major root respiratory cost [19, 46], the finding that root respiration in some halophytes declines with increasing salinities within the optimum NaCl range [47, 48] supports to this hypothesis.

Do Chloride Transporters Differ Between Halophytes and Non-Halophytes?

The mechanisms of Cl^- uptake at the root PM in halophytes remain to be discovered. In non-halophytes, the molecular identity of transporters likely to catalyse Cl^- transport at the root PM has recently been revealed (Box 1) and these putative Cl^- transport systems could also occur in halophytes. However, the picture is far from complete. More in-depth phylogenetic and functional analyses of these transporter/channel families are required both in halophytes and non-halophytes. Recent evidence indicates that single amino acid polymorphisms in these transporter genes in halophytes could explain the altered transport functions and activities compared with their non-halophytic counterparts. In the bladder cells of the halophyte *Chenopodium quinoa*, the vacuolar anion transporter *CqClC-c* has strong Cl^- selectivity, in contrast to its non-halophytic ortholog *AtClC-a* [33]; in *Arabidopsis*

thaliana AtClC-a mediates NO_3^- transport across the tonoplast, with a high selectivity for NO_3^- over Cl^- [49, 50]. Interestingly, the amino acid sequences showed that, in equivalent positions of the selectivity filter of these transport proteins, *CqClC-c* contains a serine residue while *AtClCa* possesses a proline. Given that the selectivity of these ClC transporters has been correlated to the presence of serine or proline in the selectivity filter [49, 50], this serine residue in *CqClC-c* is likely to play a crucial role in the preference of *CqClC-c* for Cl^- over NO_3^-. In another example, type-1 HKT (*ThHKT1;2*) in the halophytic *Eutrema salsugineum* was found to act as a K^+ transporter in the presence of NaCl, in contrast with its highly Na^+ selective homolog, *AtHKT1*, in the non-halophytic *A. thaliana* [51, 52]. As for the above-mentioned example of the ClC transporters, this altered cation selectivity and uptake dynamics was ascribed to single amino acid differences in a crucial domain of the protein between the two species [51, 52]. Specific amino acid variations that alter protein function in wild relatives of cultivated species are already recognised as important targets for improving crop salinity tolerance (e.g., in rice [53, 54]). Thus, there is considerable scope to study the genome of halophytes to identify key allelic variations associated with their greater salinity tolerance, particularly when considering that many non-halophytic crops have close halophytic relatives. Increasing access to next generation sequencing technologies and tools also opens up new ways to pursue genomic studies of halophytes other than *Eutrema* species; *Eutrema* has the drawback that its salinity tolerance is substantially lower than that of most other halophytes and is based on avoidance of salt accumulation [55, 56] rather than on the ability to tolerate high tissue Na^+ and Cl^- and maintain growth when under high external NaCl.

How Is Chloride Transport Regulated?

Compared to their non-halophytic relatives, halophytes have enhanced constitutive expression of specific gene families involved in ion transport (e.g., *NHXs, HKTs, SLAHs, AHAs* [57-60]). Nevertheless, salt-induced changes in protein activity are not always associated with changes in **transcriptional regulation**, emphasising the important role of post-translational regulation [33, 35, 39, 57, 58]. Anion transport is controlled by many cytosolic factors [61, 62] and it is plausible that the key anion regulators, for example $[Ca^{2+}]_{cyt}$, pH and reactive oxygen species, could play roles in the differential regulation of Cl^- transport in halophytes, particularly because their

salt-induced kinetics and modulation are substantially different from those in non-halophytes [63].

Of special interest is cytosolic Ca^{2+} signalling. Compared to other eukaryotes, plants possess a greater number of Ca^{2+} decoder proteins, characterised by a greater Ca^{2+}-specificity [64]. In particular, the **calcineurin B-like protein (CBL) and the CBL-interacting protein kinase (CIPK) network** has emerged as a key regulator of plant ion transport systems [65-67]. Comparative studies between halophytes and their close non-halophytic relatives reveal that the *CIPK* gene family is larger in halophytes, with some halophytic *CIPKs* showing salt-enhanced transcription [59, 68]. Overexpression of a CIPK from the halophyte *Hordeum brevisubulatum* (*HbCIPK2*) in *sos2-1* mutant and wild type *A. thaliana* demonstrated the importance of this kinase in ion homeostasis (Na^+, Cl^- was not measured) under salt stress; it rescued hyper-sensitivity to NaCl in the *sos2-1* mutant and further enhanced salt tolerance in wild type plants. This improvement was achieved via coordinated changes in gene expression and a possible interaction of the kinase with Na^+/H^+ transporters, K^+ transporters, and H^+ pumps [69]. Although no data are available on the role of the CIPK/CBL complexes in Cl^- transport, it can be envisaged that they might modulate Cl^- transport and its differential regulation between halophytes and non-halophytes (Fig. 3). The role of CIPKs and CBLs in NO_3^- transport [70], together with the fact that NO_3^- and Cl^- share similar transport pathways [31, 71] (Box 1), and that some halophytes can maintain NO_3^- uptake at high salinities [72], make our model plausible. This model provides a hypothesis that requires experimental testing in both halophytes and non-halophytes.

Concluding Remarks and Future Perspectives

Regulation of Cl^- uptake and translocation in plants is a significant issue, even more so under saline conditions where Cl^- toxicity potentially comes into play. We argue here that, under saline conditions, halophytes: (i) require Cl^- as an osmoticum for cell turgor and growth, (ii) invest metabolic energy for Cl^- uptake by roots, and (iii) regulate Cl^- influx rather than relying on efflux to tightly control net root Cl^- uptake. The ability of halophytes to limit Cl^- influx over the long term under high salinities has dual benefits: (i) maintains the hyperpolarised membrane potentials, becuase if a high rate of Cl^- influx via the $Cl^-/2H^+$ symporter did occur this would place an acid

load on the cytoplasm and depolarise the PM [8] (Figure 3), and (ii) reduced energy requirements not only for Cl^- but also for Na^+ transport because Cl^- influx must be coupled with cation influx (i.e., Na^+ under saline conditions) to maintain charge balance.

If halophytes regulate Cl^- influx rather than relying on efflux for determining net root uptake, would a reduction of the key transporters catalysing Cl^- influx at the root PM be a potential breeding target in the development of salt-tolerant crops? Answering this question is yet not possible but should be carefully considered. Compounding the severe physiological dysfunctions induced by high Cl^- that affect the yield and edibility of non-halophytic crops [4, 13, 14], a reduction in these transporters could potentially lead to additional nutritional deficiencies because Cl^--permeable proteins are also involved in the acquisition of other essential nutrients (e.g., NO_3^-, SO_4^{2-}, and K^+; Box 1). Employing recent advances in gene editing techniques to increase anion transporter selectivity for NO_3^- and/or SO_4^{2-}, rather than for Cl^-, should also be considered. In this context, it is important to address whether the broad substrate selectivity of Cl^--permeable proteins is behind the reported negative correlation between Cl^- accumulation and the limited salinity tolerance of non-halophytic crops. Indeed, because domestication processes of crops have been driven by targeting yield under optimal (i.e., unstressed) conditions, the selection for improved nutrient uptake may have inadvertently resulted in higher Cl^- uptake rates under high salinities, given the broad substrate selectivity of the associated transport proteins. Over time, this would result in undesirable accumulation of Cl^- in the shoots, causing potential toxicity to cellular functions. An important question arising is whether halophytes control Cl^- influx over the long term only by regulating the selectivity of the Cl^--permeable proteins or whether a lower density (i.e., lower transcript and thus lower protein levels) of these proteins in the PM also plays a key role?

Testing our hypothesis on the role of Cl^- influx in controlling root net Cl^- uptake in halophytes will require a more targeted comparison between halophytes and non-halophytes. The results will address the functional roles of Cl^- and its putative cycling at the root PM (efflux/influx) in determining salt tolerance and whether this differs between species across the salt tolerance continuum. Moreover, the potential role of CBL and the CBL-Interacting Protein Kinase (CIPK) network as possible regulator of

Cl^- transport systems in plants should also be resolved. Finally, comparative studies will also be necessary to provide insights into the economics of salt tolerance and the limits of the different strategies ('exclusion' vs. tissue tolerance) across the tolerance continuum. Indeed, because ion transport processes are not only linked to the activity of transport proteins but also to the energy status of the cell, this is of fundamental importance in breeding for salt tolerance. Implementing specific traits that operate in salt-tolerant species without consideration of the energetic costs and/or nutritional trade-offs could ultimately prove unproductive.

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Figure captions

Figure 1. Halophytes Have a More Stable Internal Shoot Cl^- Concentration with Increasing Soil Salinity, Suggesting Greater Control of Net Cl^- Uptake from the Saline Soils than for Non-Halophytes. Compared to non-halophytes (A), halophytes accumulate far greater amounts of Cl^- in shoots at low salinity and in their optimum range (D). For comparative studies see [35, 73]. This implies greater control by halophytes of Cl^- uptake from saline soils. As shown for Na^+ transport in salt-sensitive non-halophytes [42], following an increase in the external soil salinity, it is likely that salt-sensitive non-halophytes (B-C) limit net Cl^- uptake, thereby limiting shoot Cl^- concentrations increase in the short-term. Plants must largely rely on compatible solutes for osmotic adjustment during the initial period. However, over the long term, as the available energy is progressively depleted, roots cannot prevent/control net Cl^- uptake. This results in progressive increases in shoot Cl^- concentrations, leading to possible Cl^- toxicity [73]. By contrast, halophytes follow a contrasting strategy (D-E). Following an increase in the external soil salinity, halophytes modulate net Cl^- uptake in the short-term to rapidly increase shoot Cl^- concentrations. This fulfils the demands of the plant for osmotic adjustment and optimal plant growth (text for details and [74] for data). Once this "optimal" shoot Cl^- concentration is reached, halophytes are likely to modulate net Cl^- uptake to limit Cl^- translocation to the shoot and thus maintain relatively stable shoot Cl^- concentrations over the long-term [73, 75, 76].

Figure 2. Nernst Potential (E) for Passive Equilibration of Cl^- (E_{Cl}) across the Plasma Membrane with Increasing Cytosolic Cl^- Concentrations ($[Cl^-]_{cyt}$) at Three Different External NaCl Concentrations (200, 400, and 600 mM NaCl). These calculations were made based on $[Cl^-]_{cyt}$, external Cl^- concentrations, and membrane potentials (MP) in roots of halophytes grown in 200 to 600 mM NaCl. Red asterisks indicate $[Cl^-]_{cyt}$ and root cell MP in: (A) *Suaeda maritima* roots exposed to 200 mM NaCl ($[Cl^-]_{cyt}$ values measured in mature root cortical cells [77]); (B) *Atriplex amnicola* roots exposed to 400 mM NaCl ($[Cl^-]_{cyt}$ values measured in root cells with little vacuolation [78], which can be used to estimate $[Cl^-]_{cyt}$ [79]); and (C) *Atriplex spongiosa* roots exposed to 600 mM NaCl ($[Cl^-]_{cyt}$ calculated from counts measured in X-ray microanalysis using a ratio of counts to concentration of 10 [80]). The MP values under steady state salinity are from [81]. The blue shape indicates possible $[Cl^-]_{cyt}$ and root MP

following a sudden increase in NaCl concentration or salt shock treatment. The $[Cl^-]_{cyt}$ is assumed to be similar to pre-treatment conditions (in non-saline conditions, $[Cl^-]_{cyt}$ has been found to vary from 2 mM to up to 50 mM [12, 37, 82]). The values for root MP under transient salinity are from [39]. Data indicate that, in contrast to non-halophytes, halophytes can rapidly (<24 h) recover root MP following a sudden increase in NaCl concentration (or salt shock treatment) to values close to those measured under pre-treatment conditions [40, 41]. Based on the measured $[Cl^-]_{cyt}$ and the calculated E_{Cl}, passive uptake in (A), (B) and (C) would only occur with a root MP ≤ -11 mV, ≤ -20 mV, and ≤ -55 mM, respectively.

Figure 3. A Hypothetical Model Depicting How CBL/CIPK Could Alter Cl^- Influx via a High-Affinity, Proton-Coupled, Cl^--Selective Transporter (e.g., NPF6.4 [31]) in Halophytes Following Salt Stress. (A) Before salt stress and with a low Cl^- external concentration, the transporter is a high-affinity Cl^- transporter, displaying strong selectivity for Cl^- over NO_3^-. (B) Following salt stress, salt sensing at the plasma membrane (PM) and Na^+ transport across the PM results in (1) rapid elevation of the cytosolic free Ca^{2+}, and (2) CBL senses this Ca^{2+} elevation in the cytosol ('signature'), and activates CIPKs. These then phosphorylates the PM H^+-ATPase and the high-affinity Cl^- transporter. The phosphorylation of the high-affinity Cl^- transporter disrupts its high-affinity Cl^- transport activity, thus changing its substrate selectivity and limiting Cl^- competition with NO_3^-, thereby increasing NO_3^- uptake. At the same time, phosphorylation of the PM H^+-ATPase stimulates its activity. This (3) increases the cytosolic pH and (4) stimulates the activity of the Cl^- transporter. (5) The activity of the $Cl^-/2H^+$ symporter places an acid load on the cytosol (as a result of >1 H^+ per 1 Cl^- imported) and the cytosolic pH returns to a level close to the initial pH value [83]. Other low and high affinity NO_3^- transport pathways, which are also regulated by the CBL/CPK pathway (reviewed in [84, 85]) and facilitate NO_3^- uptake, are not shown in the figure.

Box 1. Chloride transport proteins at the root plasma membrane

Over past few years, exciting breakthroughs have emerged from the long-awaited discovery of genes encoding Cl^--permeable transport proteins at the root PM under *quasi* steady-state conditions. Wen *et al.* [31] presented the first molecular characterisation in *Zea mays* of the root $Cl^-/2H^+$ symporters that were proposed to catalyse root Cl^- influx in the early 1990s [37]. The two NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTERS (NPF) characterised, ZmNPF6.4 and 6.6, are homologues of the well-known dual affinity nitrate transporter AtNPF6.3 in *Arabidopsis thaliana*. Both were found to be pH-dependent NO_3^- transporters with the capacity to transport Cl^-, but with contrasting affinities. Whereas ZmNPF6.4 facilitated Cl^- transport via a saturable **high-affinity transport system (HATS)**, ZmNPF6.6 had only a **low-affinity transport system (LATS)** for Cl^-. By contrast, when supplied with NO_3^-, ZmNPF6.6 switched to a HATS NO_3^- transporter while ZmNPF6.4 changed to a LATS NO_3^- transporter with efflux activity.

Another PM-localised NPF transporter, AtNPF2.5, was also proposed to facilitate Cl^- efflux from *Arabidopsis thaliana* roots [9]. The electrophysiological features of AtNPF2.5, coupled with the findings that it is salt inducible and predominantly expressed in root cortical cells, further support its role in facilitating root Cl^- efflux. Also predominantly expressed in mature root tissues is the *Zea mays* PM ALUMINIUM-ACTIVATED MALATE TRANSPORTER ZmALMT1 [86]. This transporter mediates selective anion (SO_4^{2-}, Cl^- and NO_3^-) efflux and/or influx and has been proposed to be involved in mineral nutrition and ion homeostasis.

Other candidates are the CATION CHLORIDE COTRANSPORTERS (CCCs, $2Cl^-:Na^+:K^+$ symporter). It was recently shown in *Oryza sativa* that CCC1 is present in all root cells, mainly at the PM, and are required for cell elongation and osmoregulation through Cl^-, K^+ and Na^+ homeostasis [87]. Furthermore, in the halophyte *Suaeda maritima* grown with 150-200 mM NaCl, 100 μM bumetanide, a blocker of CCC proteins in humans, halved root Na^+ concentrations [88]. It is therefore tempting to speculate that CCCs could catalyse Cl^- transport at the root PM. Nevertheless, this hypothesis is hampered by uncertainties regarding the PM localisation of CCCs [87, 89, 90] and the use of bumetanide; this inhibitor could potentially end up targeting other ion transport systems in plants [89].

Glossary

Active ion transport: movement of ions across a membrane against the electrochemical gradient, thus requiring energy. Passive ion transport does not require energy input.

Calcineurin B-like protein (CBL)–CBL-interacting protein kinase (CIPK) network: a signalling network that acts in diverse plant stress responses. In plants, different environmental stimuli generate specific Ca^{2+} signatures. The CBL proteins are one of the plant sensors that perceive these Ca^{2+} signatures and then interact with and activate CIPKs, subsequently initiating various cellular responses.

Electrochemical difference (or gradient): the electrochemical gradient for an ion consists of electrical and chemical parts or transmembrane electrical potential difference and a concentration difference, respectively.

Equilibrium potential: for each ion, the equilibrium (or reversal) potential is the membrane potential at which there is no net flux of that ion across the membrane.

Exclusion: the ability of plants to exclude Na^+ and Cl^-, particularly from leaves, and rely on organic solutes to osmotically adjust and maintain turgor when faced with hyperosmotic conditions in saline soil solutions.

Halophyte: naturally 'salt-loving' plants, which can complete their life cycle in NaCl-rich environments (200 mM and above) where most other plants (i.e. non-halophytes) cannot survive. Halophytes are stimulated by the addition of salts, displaying a growth maximum in the 200-500 mM range, whereas for non-halophytes there is always a gradual decrease in growth upon increase of salinity.

High-affinity transport system (HATS): in this transport mode, characterised by saturable kinetics at low substrate concentrations (<1 mM), the transporter has a high binding affinity for the substrate.

Hyperaccumulation: high accumulation of ions in plant leaf tissues relative to low external concentrations.

Low-affinity transport system (LATS): in this transport mode, characterised either by saturable or linear kinetics in the high concentration range (> 1 mM), the transporter has a low binding affinity for the substrate.

Osmoticum (plural osmotica): an ionic or non-ionic solute that increases the osmotic potential of a cell.

Symporter: a transport protein that belongs to the broad category of co-transporters, or the secondary active transport mechanisms in a cell. The uphill movement of an ion is driven by the passive movement of another ion down its electrochemical gradient.

Tissue tolerance: the ability to accumulate sufficient Na^+ and Cl^- to osmotically adjust and maintain cell turgor in saline environments. This is generally achieved by sequestering toxic ions into the vacuoles away from ion-sensitive cytoplasmic enzymes. Halophytes have also evolved the ability to either sequester cytotoxic ions in specialised external structures, such as salt bladders, or to directly secrete these ions out of the shoot via salt glands.

Transcriptional regulation: regulation of the conversion of DNA to RNA (transcription) to finely tune, in time and space, the amount of RNA produced thereby controlling gene activity. By contrast, post-translational modifications occur after protein biosynthesis.

Transport protein: a protein involved in the movement of molecule (ions, small molecules or macromolecules) across a membrane. They can either facilitate diffusion (i.e., passive transport) through channels or actively transport ions against their electrochemical gradient by either co-transporters or pumps.

Highlights

- Interest in the Cl^- aspect of salinity tolerance has traditionally focused on non-halophytes.
- Knowledge of Cl^- regulation in 'salt-loving' halophytes is limited even though these plants thrive and survive at much higher external Cl^- than non-halophytes and use Cl^- for osmoregulation.
- Proteins catalysing root Cl^- transport have recently been characterised in non-halophytes, but this knowledge needs to be extended to halophytes. Of interest is that single amino acid polymorphisms in halophytic transporters alter their function compared to the non-halophytic homologues.
- Our understanding of post-translational regulation of anion channels in stomata is fairly advanced, but that of root Cl^- transport remains elusive.
- The calcineurin B-like protein (CBL)-CBL-interacting protein kinase (CIPK) network regulates several ion transport systems in plants. Does it also modulate Cl^- transport?

Outstanding questions

- Why do some plant species possess high sensitivity to elevated Cl^- levels whereas others suffer predominantly from Na^+ toxicity? What are the targets of high cytoplasmic Cl^- concentrations, and does enzymes tolerance to high Cl^- vary between halophytes and non-halophytes?
- Although based on a limited number of studies, patch-clamp experiments have found that monocots have a greater number of anion currents compared to the dicot Arabidopsis. Is this difference casual or is there an intrinsic difference in the Cl^- transport pathways in monocots and dicots?
- What is the magnitude and importance of Cl^- efflux in different species across the salt tolerance continuum and to what extent does this Cl^- cycling (influx vs. efflux) at the root PM relate to salt tolerance?
- Because salinity in the field is highly heterogeneous in time and space, how does this heterogeneity affect the expression and regulation of Cl^- transporters and Cl^- uptake within roots exposed to different salinities? What feedback mechanisms regulate Cl^- uptake in roots (localised, i.e. root Cl^- concentrations, or systemic, e.g., shoot Cl^- concentrations) under uniform and heterogeneous salinities?
- What are the second messengers that regulate the activity of transporters determining Cl^- influx/efflux in roots? Do they have a similar effect on halophytes and non-halophytes?
- Are there different proteins that act as intermediaries in the regulation of Cl^- transport proteins at the root PM by cytosolic messengers? For example, it has recently been shown that aquaporins are involved in ABA-mediated stomatal closure. Could the same occur at the root PM? Does the CBL/CIPK network regulate Cl^- transport in roots?
- What controls the dual/multiple transport ability in anion-permeable proteins and are they differentially controlled between halophytes and non-halophytes?

FIGURES

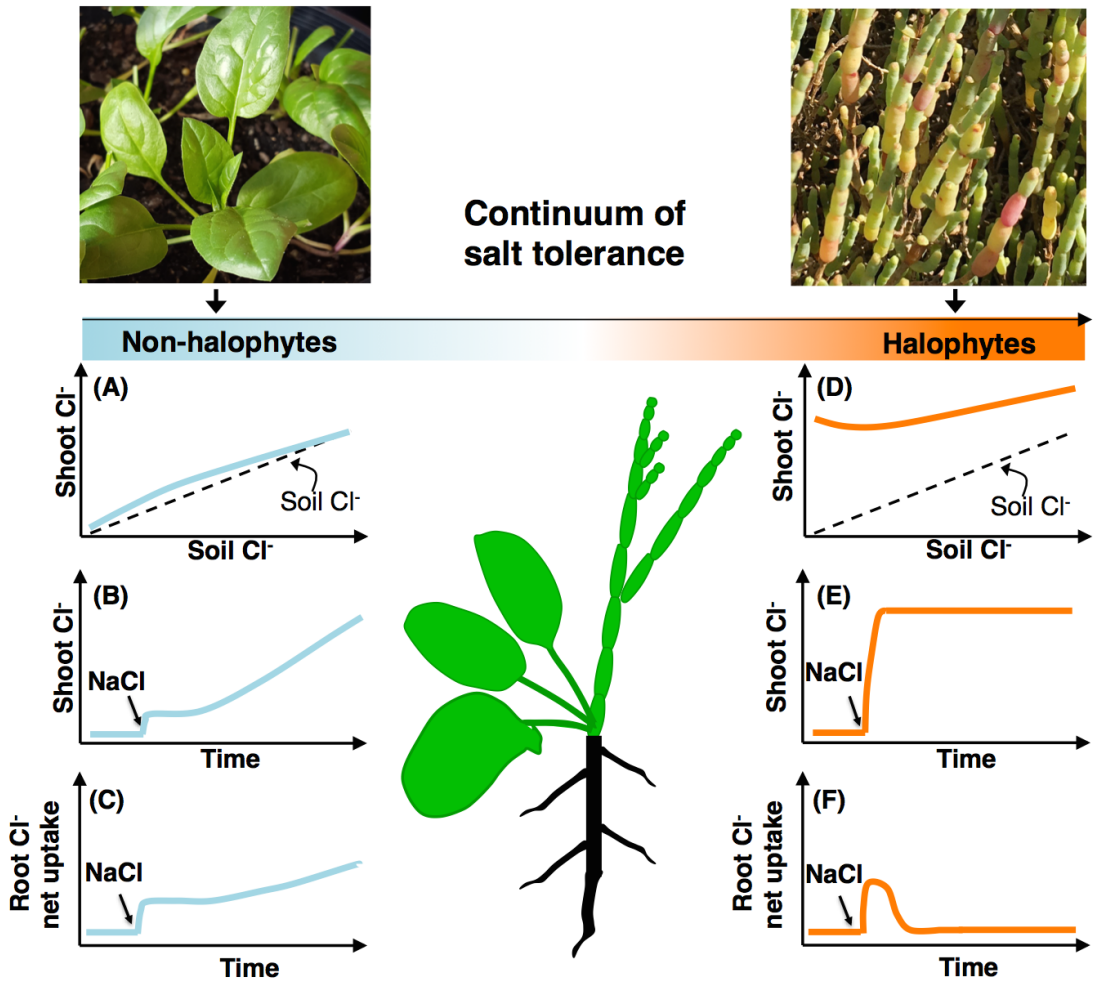

FIGURE 1

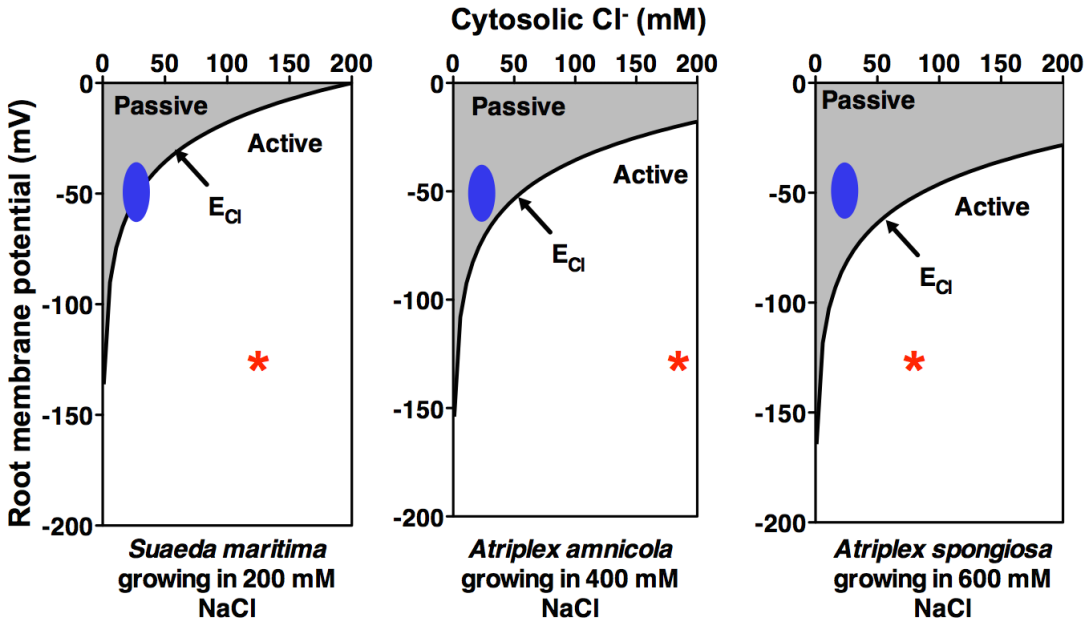
Cytosolic Cl- (mM)
Root membrane potential (mV)
0
50
100
150
200
-50
-100
-150
-200
Passive
Active
ECl
Suaeda maritima
growing in 200 mM
NaCl
Atriplex amnicola
growing in 400 mM
NaCl
Atriplex spongiosa
growing in 600 mM
NaCl

FIGURE 2

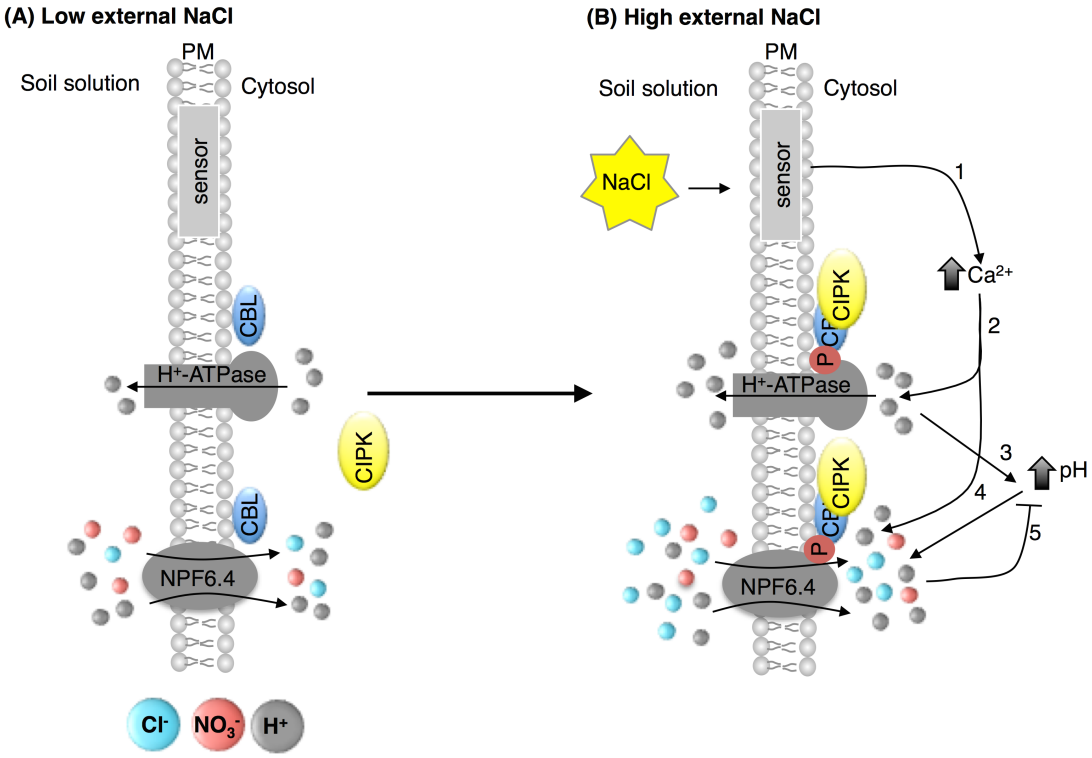
(A) Low external NaCl
PM
Soil solution
Cytosol
sensor
CBL
H+-ATPase
CBL
NPF6.4
CIPK
Cl-
NO3-
H+
(B) High external NaCl
PM
Soil solution
Cytosol
NaCl
sensor
1
Ca2+
2
CIPK
P
CBL
H+-ATPase
3
pH
4
CIPK
P
CBL
5
NPF6.4

FIGURE 3