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Chloride on the move

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Abstract

Chloride (Cl^-) is an essential plant nutrient, but under saline conditions can accumulate to toxic levels in leaves; limiting this accumulation improves the salt tolerance of some crops. The rate-limiting step for this process – the transfer of Cl^- from root symplast to xylem apoplast, which can antagonize delivery of the macronutrient nitrate (NO_3^-) to shoots – is regulated by abscisic acid (ABA) and is multigenic. Until recently the molecular mechanisms underpinning this salt tolerance trait were poorly defined. Here, we discuss how recent advances highlight the role of newly identified transport proteins, some that directly transfer Cl^- into the xylem, and others that act on endo-membranes in 'gatekeeper' cell types in the root stele to control root-to-shoot delivery of Cl^-.

Chloride - a problem nutrient?

Chloride (Cl^-) is a plant nutrient with proposed regulatory roles in photosynthesis, transpiration, fertilization, nutrition and growth; how the accumulation of Cl^- and its movement across membranes influence these key plant physiological processes via changes in membrane potential, enzyme stability, charge balance, pH, osmoregulation, volume control and turgor have been discussed elsewhere [1-3]. As it is a charged solute, Cl^- moves into cells and between cellular compartments predominantly through ion transport proteins (**transporters**) embedded within cellular membranes. This occurs either passively down its difference in electrochemical potential through ion channels or actively through carrier proteins such as **symporters** or **antiporters** that can move Cl^- using the difference in electrochemical potential for another ion (such as protons, H^+). Over the past ten years, and particularly the last two, a number of plant transport proteins permeable to Cl^- have been identified and characterized, which has advanced our knowledge of plant Cl^- transport from the biochemical, physiological and electrophysiological level into the molecular domain. Outlined in Figure 1 are major transport steps across cellular membranes that affect nutritive Cl^- uptake, translocation and storage. The key rate-limiting '**gatekeeper**' step modulating Cl^- accumulation in the shoot has been shown to be the loading of Cl^- from the root **stelar symplast** into the xylem **apoplast**, which is regulated following drought and **salinity** stress via abscisic acid

(**ABA**) [2, 4-10]. In the root, ABA inhibits xylem loading of Cl⁻, but ABA has no effect on its uptake [7, 9]. This leads to accumulation of Cl⁻ in the root under saline conditions, contributing to shoot salt exclusion. Whilst other processes such the regulation of root epidermal uptake or compartmentation of Cl⁻ in the root vacuole are acknowledged to affect shoot Cl⁻ accumulation they have been reported to vary less between the materials examined, and are not the key process regulated by ABA [1, 2].

In **saline** conditions, limiting accumulation of Cl⁻ in leaves is a sub-trait of salt (NaCl) tolerance and is a **multigenic** process [2] (Fig 1). However, the molecular factors that limit long-distance Cl⁻ transport have been relatively neglected compared to those for the other ionic component of salt, sodium (Na^+) [11]. Here we review the latest findings related to the regulation of Cl⁻ transport from root to shoot, including an overview of newly identified Cl⁻ transporting proteins associated with the root **stele**. This includes proteins that directly catalyze the movement of Cl⁻ from the root **symplast** to the xylem [12-14], and several endo-membrane transport proteins that appear to influence long-distance Cl⁻ transport and salt tolerance [15-18]. We evaluate these studies and propose potential future research directions for studies with the aim of improving Cl⁻ exclusion of Cl⁻ sensitive plants [19]. Although the scope of this review is mostly limited to the root **stele**, we discuss the similarities and differences in Cl⁻ transport processes with stomatal guard cells. We do this to emphasize two important concepts that highlight the plants' flexibility in its transport regulation: (i) plant cells appear to repurpose particular proteins in different tissues to perform novel roles; and, (ii) plants use closely related proteins in different ways in different cell types.

Chloride – a neglected component of salt toxicity?

Salinity-induced yield reductions in our conventional staple or high value crops are common in coastal and arid regions, and are substantial and increasing for irrigated agriculture [1]. It is estimated that up to 8% of rain-fed (dryland) agriculture and 20% of irrigated agriculture is currently affected by **salinity**, with both figures expected to double by 2050 [20]. **Saline** soils are more prevalent in arid and semi-arid countries.

For instance, in Australia over 67% of its cropping region has the potential to develop **salinity** issues in any one season [20]. There are at least three sub-traits that confer **salinity** tolerance – **leaf salt exclusion**, **tissue tolerance** and **osmotic adjustment** – each sub-trait is under the influence of multiple genes acting on multiple underlying components [11, 21, 22].

To date, Na^+ is the ion that has most commonly been associated with crop yield reductions due to salt accumulation in the soil solution [11]. In fact, Na^+ and salt are often used interchangeably in the literature. However, in woody perennial crops such as grapevine (*Vitis* spp. L.), citrus (*Citrus* spp. L.), and avocado (*Persea americana* L.*)*, and legumes such as soybean (*Glycine max* L.) and faba bean (*Vicia faba* L.) it is the accumulation of Cl^- in leaves, not Na^+, that is often best correlated with decreased transpiration, photosynthesis, crop yield and quality – and eventually plant death [2, 5, 6, 10, 23-26]. This association exists not because Cl^- is metabolically more toxic than Na^+ to these species, but because they are able to secrete a greater proportion of their Na^+ in roots and/or woody stems. This limits Na^+ transport to the leaves to reduce its potential impact upon cellular metabolism within photosynthetic organs [2, 21]. For instance, trifoliate orange (*Poncirus trifoliata* L.) was able to maintain shoot Na^+ exclusion in treatments below 100 mM by secreting it into the woody tissue of roots and the basal stem, presumably through xylem retrieval, whereas leaves accumulated high concentrations of Cl^- even when under a 25 mM NaCl treatment [27]. It is now emerging that even cereals classically thought to be Na^+ sensitive are also sensitive to shoot Cl^- accumulation (see below).

In horticulture, the scion of salt-sensitive species such as grapevine and citrus are grafted to **rootstocks** that limit the delivery of Cl^- to the root xylem (e.g. trifoliate orange) to confer a degree of shoot salt exclusion and improve growth and yield in saline environments [6, 10, 25, 26]. Comparative genomics of these **rootstocks** is being used to uncover the molecular determinants that control the delivery of Cl^- to the shoot [6, 28]. The hope is that this will allow manipulation of specific Cl^- **transporters** to improve crop salt tolerance as has occurred for the manipulation of Na^+ transporters [19, 29-31].

There are likely to be multiple molecular targets of Cl^- (and Na^+) toxicity, most of which are unknown [11]. For Cl^-, some are likely to occur via interference in its nutritive roles. All that has been observed so far are the phenotypes associated with salt accumulation. During salt stress, the effects of Cl^- can be additive and/or synergistic to Na^+. Treatments of NaCl can affect the growth and physiology of a variety of species more than treatments that contain only high concentrations of one of salt's constituent ions (Cl^- or Na^+). This occurs even in wheat, barley and rice that are classically thought to be more Na^+ sensitive [32-36]. Whilst it is difficult to separate Cl^- toxicity from Na^+ toxicity, and other components of salt stress, some toxic effects have been shown to be relatively Cl^- specific when using complex mixed salt solutions. For instance, in faba bean, a significant decline in leaf chlorophyll was observed following a treatment containing 100 mM Cl^- without Na^+, but not following a 100 mM Na^+ treatment lacking Cl^- [34]. It has also been shown that exposure of roots to Cl^- can inhibit gas exchange via an indirect long-distance signal, which induces an alkalinisation of the leaf **apoplastic** pH – resulting in a redistribution of leaf ABA and stomatal closure [37]. Although the interpretations of experiments that alter either Na^+ or Cl^- in isolation remain controversial, as they have to change the concentration of several counterions at the same time, these treatments are consistent with Cl^- toxicity being a significant contributor to salt stress. Such approaches could also be used to identify key players in Cl^- transport pathways and the targets of ion specific toxicity.

An important accompanying and detrimental effect of increased root Cl^- uptake, and its accumulation in shoot vacuoles, is the reduction in the uptake and storage of the major biological building block nitrogen. This well-documented effect occurs through antagonism of NO_3^- transport and accumulation of Cl^- (e.g. [12, 13, 38]). Both are monovalent anions with a similar ionic radius, can be transported through the same or different proteins (see transport selectivity section below), and both perform a role in charge balance and turgor regulation. We propose that the ratio of NO_3^- and Cl^- in the shoot may be a useful indicator of salt tolerance similar to the well described **K^+/Na^+ ratio** [2], as depression of the NO_3^-/Cl^- ratio correlates with a reduction in growth [13]. As the severity of sub-lethal Cl^- exposure increases, and Cl^- accumulates to high concentrations, marginal necrosis occurs (also known as leaf burn) [39], as well as a depression in fertilization and yield [40]. Application of NO_3^- to reduce Cl^- uptake, is sometimes successful in reducing Cl^- toxicity [39].

Getting Cl^- from root-to-shoot – what's known, what's new?

To limit accumulation of Cl^- in leaves, the net transfer of Cl^- into the root xylem should be minimized. This can occur by limiting Cl^- entry into the xylem and/or maximizing Cl^- retrieval from the xylem (Fig 1 and 2). The movement of Cl^- across the plasma membrane of the **stelar** cells into the root xylem **apoplastic** space is passive, down a difference in **electrochemical potential** for Cl^-, and thus it does not require direct expenditure of energy. Figure 2 describes the biophysics for the loading/unloading of Cl^- into xylem vessels. It was hypothesized that plants down-regulate the activity and expression of Cl^- transporters that load Cl^- into the xylem during salt exposure, or following drought, via an ABA mediated pathway [9]. Such a phenomenon is known to occur for K^+ loading via the **SKOR** channel during water stress [41-43], and this putative property has been used as a framework to identify proteins that regulate loading Cl^- into the xylem. In non-stressed conditions, there is an electrochemical potential difference for protons between the root **symplast** and the xylem-associated **apoplast**, so it is possible that a Cl^-/H^+ **symporter** could actively retrieve Cl^- from the xylem **apoplastic** space back into the root symplast, if present on this membrane. Thermodynamically, this action against the **electrochemical potential** difference for Cl^- could resemble the $Cl^-/2H^+$ symport described for the initial uptake of Cl^- into root hair cells [44] (Fig 1). As such, this would be less energy efficient than reducing the passive movement of Cl^- into the xylem apoplast as it requires direct use of some of the difference in electrochemical potential for H^+, built up by the H^+-ATPases on the plasma membrane of these cells. Retrieval of Cl^- from the root xylem through the direct action of a **CCC** has been proposed [45], although others have shown this is an endo-membrane protein so is not likely to be directly involved [16] (*see **CCC** section below*). Furthermore, recent evidence has been used to suggest that the disruption of cytosolic Cl^- through knockout of vacuolar Cl⁻ transporters has knock-on effects on root-to-shoot transfer of Cl⁻ [18] (*see **ALMT9** section below*).

In the following section, we highlight proteins implicated in modulating shoot Cl⁻ accumulation either through facilitating transfer of Cl⁻ transport to the root xylem or the regulation of this process (Fig 3), we also comment on several further candidates that are yet to be examined for their role in long-distance Cl⁻ transport (Fig 3).

Nitrate transporter1/Peptide transporter (NPF) proteins

A recent microarray screen identified the first protein proposed to directly catalyze Cl⁻ transport into the root xylem, *Arabidopsis thaliana* L. (arabidopsis, At) AtNPF2.4 [14]. Protoplasts were specifically isolated from the stele or epidermis/cortex to probe for transcripts that: 1) were expressed preferentially in the stele; 2) had their expression negatively regulated by ABA and NaCl; and 3) were likely to encode an anion transport protein, as it was deemed from previous literature that these were properties that transporters controlling root-to-shoot transport of Cl⁻ may possess [14]. Only two genes fulfilled these criteria, *AtNPF2.4* and *AtNRT1.5/AtNPF7.3,* with the former being chosen for further characterization. The *AtNPF2.4* promoter drove expression specifically within root stelar cells, the protein was localized to the plant plasma membrane, overexpression of *AtNPF2.4* resulted in a 23% increase in shoot Cl⁻, and when *AtNPF2.4* was expressed in *Xenopus laevis* oocytes it catalyzed Cl⁻ efflux at membrane potentials equivalent to those in the stele (around -120 mV) [14]. The currents ascribed to AtNPF2.4 were channel-like and were not pH-dependent, but were unlike the major conductance thought to be responsible for xylem loading of NO_3^- and Cl⁻ (**X-QUAC**) [9, 46, 47], they were small in magnitude, non-rectifying, did not carry NO_3^- and were dependent upon external K^+ or Na^+ [14]. This means that AtNPF2.4 does not encode an **X-QUAC** type channel, or that specific regulatory factors not present in oocytes are required for it to function as it does in the plant (e.g. kinases/phosphatases). Alternatively, arabidopsis may not contain **X-QUAC**, with no equivalent experiments to that in barley and maize being yet conducted [9, 46, 47]. Regardless, it is clear that loading of Cl⁻ to the arabidopsis xylem is a multigenic trait (as it appears to be in grapevine [10, 26, 28], maize [9] and barley

[46, 47], see Figure 3) as silencing of *AtNPF2.4* resulted in only a ~20-30% reduction in shoot Cl^{-} concentration [14].

AtNPF2.4 is a member of NAXT subfamily (Nitrate excretion transporters) (7 members) named after AtNAXT1/AtNPF2.7, a root NO_3^{-} efflux transporter [48]. The Cl^{-} transport activity of AtNPF2.4 indicated the potential involvement of other root specific NAXTs in Cl^{-} excretion from the root (Fig 3). In that regard, it would be instructive to test the expression profiles of the NAXTs of unknown function for their root tissue localization and their regulation by salt.

The *NPF* gene family in arabidopsis encodes several other candidate proteins for Cl^{-} transport to the stele. The expression of some of these genes is regulated by salt stress, and some have a demonstrated role in affecting NO_3^{-} distribution among tissues [48-53]. Amongst these, the stelar-specific *AtNRT1.5/AtNPF7.3* was the only other transcript to encode an anion transporter identified in the microarray screen described above [14]. This protein was designated as one of the transporters that loads NO_3^{-} directly into the root xylem and affects its delivery from root-to-shoot [49]. Salt stress significantly down-regulates *AtNPF7.3* expression [51], which might be a cause, or be a consequence of, the antagonism between Cl^{-} and NO_3^{-} shoot accumulation. Interestingly, knockout of *AtNPF7.3* resulted in greater salt tolerance, which was attributed to an increase in root NO_3^{-} content [51]. AtNRT1.8/AtNPF7.2 is a designated retriever of NO_3^{-} from the root xylem, its expression is significantly induced by salt stress and overexpression increases salt tolerance [50]. It would be interesting to examine root and shoot NO_3^{-}/Cl^{-} ratios in the *Atnpf7.2* and *Atnpf7.3* mutants and corresponding overexpression plants. AtNPF7.3 may also facilitate Cl^{-} transport *in planta* and its loss may reduce shoot Cl^{-}, although this remains to be tested. Similarly, increased AtNPF7.2 abundance may increase Cl^{-} retrieval from the xylem, energized by the difference in electrochemical potential for H^{+}, and reduce shoot Cl^{-} accumulation (Fig 2 and 3). However, it is known that *AtNPF7.2* overexpression and *AtNPF7.3* knockout also results in improved tolerance to biotic, cadmium, cold and osmotic stresses, so increased NO_3^{-} accumulation in roots may simply provide a general increase in stress tolerance [54].

Slow-type anion channel associated homolog 1 (SLAH1)

In guard cells of arabidopsis, the Slow-Type Anion Channel-associated 1 protein (AtSLAC1) is responsible for the major component of anion efflux (Cl^- and malate), important for stomata closure [55, 56]. The AtSLAC1-homolog 1 (*AtSLAH1*) is only expressed in root pericycle cells [56]. Two recent reports indicate that AtSLAH1 has a major role in shoot accumulation of Cl^- [12, 13]. Overexpression of *AtSLAH1* either specifically in the root stele, or constitutively, led to increased shoot Cl^- accumulation and decreased salt tolerance [13], and knockdown or knockout of *AtSLAH1* reduced shoot accumulation of Cl^- by about 30% [12, 13]. AtSLAH1 is electrically silent when expressed by itself in *X. laevis* oocytes [12, 13], as are AtSLAC1 and AtSLAH3, but pore mutations that make AtSLAC1 and AtSLAH3 constitutively active in oocytes do not work for AtSLAH1 [12]. It was hypothesized that AtSLAH1 is a silent subunit and does not transport Cl^- directly [13]; instead AtSLAH1 interacts with AtSLAH3 to seemingly changes AtSLAH3 transport properties increasing its capacity to transport Cl^- [13]. AtSLAH3, is expressed in the pericycle and stomatal guard cell and is much more selective for NO_3^- over Cl^- when activated by phosphorylation with Ca^{2+} dependent kinase 21 (AtCPK21), as occurs in stomatal guard cells [12, 57, 58]. It was shown that the interaction between AtSLAH1 and AtSLAH3 was specific amongst the AtSLAC1 homologs in arabidopsis (e.g. not AtSLAC1 or AtSLAH2/4), but AtSLAH1s from other species such as medicago, poplar, and the Venus fly trap could all activate AtSLAH3 currents [12]. This work highlights that the interaction between NO_3^- and Cl^- transport is at least in part due to competition in the transport of both anions through the same set of transporters [59].

Cation-Chloride Co-transporters (CCCs)

Cation-Chloride Co-transporters (CCCs) were first characterized in animal cells where they catalyze Cl^--cation co-transport (with Na^+, K^+, or both); they regulate cellular Cl^- concentration and so influence neuronal excitability, cell volume control and osmoregulation in kidneys [60]. In animals, there are routinely multiple *CCC* genes found in one organism, in plants only one or few *CCC* representatives are commonly found per species [16, 45]. The first plant CCC characterized was from arabidopsis [45]. When expressed in *X. laevis* oocytes AtCCC co-transported Cl^-,

Na^+ and K^+, and knockout of *AtCCC* increased shoot Cl^- accumulation, whilst decreasing its accumulation in roots. Coupled to its expression in the root vasculature, this led to the conclusion that CCC may retrieve Cl^- from the root xylem [45]. Subsequently, CCC in rice, grapevine and citrus have been investigated as candidates for improving plant salinity tolerance, and their misexpression has led to altered shoot Cl^-, but the mechanism by which this occurs remains inconclusive [6, 16, 61, 62]. OsCCC was localized to the plasma membrane [61, 62]; however, it would seem pertinent to revisit this as AtCCC and the grapevine *CCC* localized to the Golgi and trans-Golgi Network (TGN) [16]. If it is an endo-membrane protein CCC is likely to affect root-to-shoot Cl^- distribution indirectly, or via a complex mechanism that is not mediated at the plasma membrane (Fig 3). *CCC* expressed in multiple tissues and knockout plants of *AtCCC* and *OsCCC* have similar severe dwarf phenotypes and low fertility in the absence of salinity, so it is clear these proteins have important functions that are unrelated to those caused by small changes in xylem Cl^- [16, 45, 62]. In rice, OsCCC was shown to be vital for cell osmoregulation and elongation through a control of cytosolic Cl^- concentrations [62], which would be another explanation for the stunted growth phenotype observed for *Atccc* plants.

Cation/H^+ exchanger (CHX)

Soybean is a moderately salt tolerant crop species with Cl^- exclusion implicated to be a major mechanism contributing to its salt tolerance [63]. A cation/H^+ exchanger (of the **CPA2** family), GmSALT3/CHX1, was shown to localize to the endoplasmic reticulum of root vasculature-associated cells, and affects Na^+ exclusion and salt tolerance of soybean [15]. GmSALT3 appears to also affect Cl^- transport to the shoot [17], which again suggests the involvement of vascular endo-membrane transporters in regulating homeostasis in xylem sap as shown for CCCs. It is unclear whether GmSALT3/CHX1 directly affects cytosolic Cl^- (and Na^+) concentration to impact plasma membrane transport as suggested for **ALMT**s (see below) as the characterized members of this family from arabidopsis are considered to be K^+/H^+ exchangers [15]. Though, this is a possibility as two CPA2 proteins from drosophila *(Drosophila melanogaster)* were recently characterized, one as a H^+-Cl^- symporter and one as a Na^+/H^+ exchanger [64]. Alternatively, the unidentified mechanism of shoot Cl^- regulation by root vascular endosomal transporters may occur through endosomal pH effects or vesicle trafficking such as those noted for the CPA1 type endosomal **NHX** proteins – that also predominantly transport K^+ [65].

Aluminum Activated Malate Transporters (ALMTs)

Aluminum Activated Malate Transporters (ALMTs) were named after the first protein cloned from this family, from wheat, which carries aluminum (Al^{3+})-activated malate efflux – this chelates Al^{3+} and confers tolerance [66]. ALMT are now known to be multigenic (with 14 in arabidopsis and 9 in rice), and most that have been characterized subsequently are not Al^{3+} activated nor have any role in Al^{3+} tolerance [67]. Instead, they play diverse physiological roles such as stomatal aperture control, anion homeostasis, fruit quality and seed development, and are widely expressed in plant tissues [68-70]. Some ALMT are permeable to Cl^-, making them good candidates for studying for a role in long distance Cl^- transport (e.g. **AtALMT9** and **AtALMT12**, Fig 3) [18, 68-71]. AtALMT9, previously characterized as carrying Cl^- into the stomatal guard cell vacuole, and for a role in facilitating stomatal opening, was recently shown to regulate long distance transport of Cl^- and Na^+ [18, 68]. In *Atalmt9* plants, Na^+ and Cl^- accumulation in the shoot decreased within a day of a 100 mM NaCl treatment but was restored to wildtype levels by 7 days [68]. It was speculated that the likely increase in cytosolic Cl^- (and Na^+) brought about by a reduced capacity for Cl^- storage in the vacuole of root stelar cells constituted a signal to increase transcription of transporters important for regulating long distance transport of Na^+, *CHX21* and *High-Affinity K^+ Transporter1* (*At**HKT1**.1)* so reducing shoot salt load [18]. Pleiotropic compensation for AtALMT9 knockout may be the reason that longer-term effects on salt accumulation are not seen. Another ALMT, the plasma membrane localized AtALMT12 is expressed in guard cells conducts cellular anion efflux (Cl^- and malate), and is a major component of the ABA-activated R-type anion current [69]. Interestingly, AtALMT12 is also found in root stelar cells like AtALMT9 [72]. Therefore, it is a good candidate for being another channel that catalyzes direct xylem loading of Cl^- downstream of ABA signaling, although its role in this process is yet to be examined.

Chloride channel (CLC)

Plant CLCs (Chloride channels) that localize to the tonoplast regulate vacuolar sequestration of Cl^- and NO_3^-, which makes them possible players in the regulation of Cl^- homeostasis. Of the seven CLCs in arabidopsis, two directly transport Cl^-, with the others transporting NO_3^- [73-78]. Tonoplast AtCLCc secretes Cl^- into root vacuoles and helps improve salt tolerance, and regulates gas exchange through its role in light induced opening and ABA-induced closure [73]; AtCLCg, localized to mesophyll tonoplast, is predicted to have a similar role for Cl^- compartmentation in leaf mesophyll as its knockout showed increased sensitivity to salt [74]. *CLC* have their expression upregulated by salt in rice, maize and citrus [79-81], and overexpression of tonoplast localized GmCLC1 in soybean increased its salt tolerance [82, 83], indicating, again that direct transfer of Cl^- is not the only factor controlling Cl^--related tolerance in plants. For further information on other CLC members, which also localize to other endo-membrane, we refer readers to [84].

ABC transporters and ICln

Multidrug-Resistance Protein 4 (MRP4) is a member of the ATP-Binding Cassette (ABC) family that was shown to be involved in S-type anion channel activity in guard cells [85]. *AtMRP4* was is expressed in primary roots and its expression is up-regulated by salt stress [85]. A functional study of the effect of AtMRP4 (or its homologs) on Cl^- transport in the root is therefore needed. Chloride-Conductance Regulatory Protein (ICln) in animal cells performs as an anion channel in artificial membranes [86]. Although microarray experiments indicated that the ICln homolog in arabidopsis (AT5G62290) did not respond to salt stress [87], in citrus, *CcICln* is differentially expressed in rootstocks with differing Cl^- exclusion capacities, suggesting the involvement of *CcICln* in Cl^- transport [6].

Transport selectivity

A molecular basis for transporter selectivity of Cl^- and NO_3^- has been revealed through the manipulation of the selectivity filter of the transport proteins **AtCLCa** and **AtSLAH2.** Single (but different) amino acid mutations in either proteins shifts the anion specificity between the two anions. This occurs through a conformational change in the transport pore (S228 in **AtSLAH2**; or P160 in **AtCLCa**), or by a mechanism likely to be related to gating (E203 in **AtCLCa)** [88-91]. Conceivably, mutagenesis of a range of endogenous transporters to improve selectivity for NO_3^- over Cl^- in the root (particularly within the stele) could reduce the shoot transfer of Cl^- and improve salt tolerance, although this could interfere with the nutritional roles of Cl^- at low salinity [3].

Transport regulation – different cells, different stories

The regulation of ion efflux from cells surrounding the xylem and stomatal guard cells is opposite (i.e. ABA activates ion efflux in guard cells but inhibits this process in xylem-associated cells). As such, it is not surprising that different transporters facilitate these processes (such as **SKOR** in the stele, versus **GORK** in guard cells – for K^+), and for those transporters that are the same in both cell types (such as AtSLAH3), a different suite of ABA regulatory proteins are likely to be present. In guard cells, AtSLAH3 is a component of the S-type anion current involved in (ABA-activated) guard cell closure [57]; recently AtSLAH3 was also shown to impede stomatal opening through inhibiting the uptake of K^+ via direct interaction with the K^+ channel **AtKAT1**, along with AtSLAC1 [92]. Therefore, it would be interesting to examine if both AtSLAH3 and AtSLAH1 interact with other proteins in root pericycle cells, and whether these are kinases such as the AtCPKs that regulate AtSLAC1 homologs in guard cells, or are other transporters.

One such candidate set of proteins is the aquaporins. In citrus genotypes, *PIP1* expression appears to be associated with Cl^- accumulation, probably due to the rate of water movement into the xylem, hydraulic conductance and transpiration [93]. Recently, a PIP2;1 aquaporin was shown to interact with OST1-SLAC1 and CPK6/23-SLAC1 complexes in arabidopsis [94]. Although this work focuses on CO_2 regulation of AtSLAC1 transport, it invites speculation that the AtSLAH1/AtSLAH3 complex may also interact with aquaporins in the xylem stelar cells, thus coupling water flow and hydraulic conductance in the xylem to Cl^- transport. AtPIP2;1 was also recently shown to transport Na^+ so the coupling of ion and water transport may occur through a single aquaporin within the stele, which could be balanced by movement of Cl⁻ [95].

Other post-translational signals, in addition to ABA, that may regulate Cl⁻ loading of the xylem include **ROS**, which was associated with Cl⁻ exclusion in soybean [96], GABA and ATP, which have been shown to inhibit ALMT activity [67, 97, 98]. ATP also regulates CLC activity so may affect root storage capacity for Cl⁻, and therefore affect delivery of Cl⁻ to the xylem [73, 99]. All three have effects in guard cells [97, 100, 101], but their effects on xylem loading are yet to be determined.

Another way ABA may differentially regulate Cl⁻ efflux across stelar and guard cell plasma membranes is by regulating transporters differentially. It was recently found that *AtSLAH1* expression was down-regulated by salt and ABA, but differences were found in regulation of *AtSLAH3* expression [12, 13]. In one study, no significant regulation of *AtSLAH3* by salt or ABA was found, which was interpreted as being a potential mechanism to sustain NO_3^- loading to the shoot and would assist in maintaining a higher shoot NO_3^-/Cl⁻ ratio [13]. However, *AtSLAH3* was found to be downregulated by ABA in another study [12], although less significantly than *AtSLAH1*. We interpret this as being due to differences in growth conditions, and propose that there is likely to be additional factors regulating the expression and activity of anion transporters in the root stele.

ABA-responsive elements were identified in the promoter region of *AtNPF2.4*, which could be explored in further detail to determine how expression of *AtNPF2.4* is downregulated by ABA. Expression of ***AtHKT1.1***, which regulates shoot Na^+ by retrieval of Na^+ from the xylem into root xylem parenchyma cells, has been reported to be downregulated by root-specific *ABA-insensitive 4* (ABI4) binding to elements within its promoter. This is particularly interesting as simultaneous Na^+ and Cl⁻ accumulation in the shoot would need to be coordinated with that of other ions to ensure charge balance (as was exemplified by the At*almt9* phenotypes). It is yet to be shown how ABA regulates ***SKOR*** or *SLAH* expression, but it plausible that there are common elements or transcription factors regulating a number of these transporters involved in shoot delivery of solutes.

Concluding remarks

Cl^- toxicity in plants is a significant issue. An important mechanism to reduce Cl^- toxicity is to reduce accumulation of Cl^- in the shoot, which requires alteration of transport processes. It appears that key **gatekeepers** of shoot Cl^- accumulation are root stelar cells, and transporters within these cells facilitate loading of Cl^-, some known and with some yet to be identified (Fig 3). Amongst these, NPF and SLAH proteins have demonstrated roles in modulating long-distance transport of Cl^- and are currently being targeted to improve Cl^- exclusion and salinity tolerance of crop plants. Endo-membrane Cl^- transporters (AtALMT9, CCCs and CHXs) are emerging with their roles in regulating long distance Cl^- transport, as well as the established role of CLCs. However, research revealing the identity of regulatory proteins for these and other stelar Cl^- transporters in response to signals such as ABA and Ca^{2+} is still at an early stage. The existence of post-translational mechanisms that affect transport activity (e.g. heterodimerisation and phosphorylation) suggests that Cl^- transport is tightly regulated for nutritional reasons that are still to be determined.

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Glossary

ABA: Abscisic Acid – is a plant hormone that plays a central role in responses to abiotic and biotic stress, e.g. salinity, drought, low temperature and pathogen attack. Salt stress triggers the synthesis and distribution of ABA throughout the plant, which induces expression of numerous salt stress-related genes, as well as a range of physiological processes, such as the closure of stomata.

Antiport(er): A type of carrier transporter that is able to transport different solutes across a membrane in the opposite direction at the same time. It is a type of co-transporter, with a similar principle of action to a symporter, except moving solutes in the opposite direction, rather than the same direction. Normally, the movement of one ion is down its difference in electrochemical potential, allowing the other ion to be pumped against its difference in electrochemical potential.

Apoplast: The volume outside the plasma membrane through which water and small molecules (such as Cl^-) can move. In the mature root, apoplastic flow is greatly slowed by the hydrophobic Casparian Strip in the endodermal cell layer.

AtCLCa: Arabidopsis Chloride Channel a, a NO_3^-/H^+ exchanger localized to the tonoplast in plants. The Thr38 residue is important for its function in stomatal closure in guard cells (Cl^- efflux).

AtSLAH2: Arabidopsis SLAC1 Homologue 2, a NO_3^- selective anion channel. This channel can become more selective for Cl^- by mutation of the Ser-228 residue.

CCC: Cation-Cl^- co-transporters, that appear to be located in the endo-membranes of stelar cells, that affect root-to-shoot transfer of Cl^-.

CPA: Cation/proton antiporter super family. It divides into family 1 and 2. CPA1 contains NHX transporters (Na^+/H^+ exchangers). CPA2 contains CHX transporters (cation/H^+ exchangers).

Electrochemical potential difference: The difference between two phases in the electrochemical potential of a particular solute, determined by the respective activities of the solutes in the two phases, the charge of the solute and, if the solute is charged, the difference in electrical potential (voltage) between the two phases.

Gatekeeper: Used to denote a process or cell type that is, or controls, a rate-limiting step (see [8]).

GORK: Guard cell K^+ outwardly rectifying K^+ channel, a protein mediating K^+ efflux out from guard cells.

HKT: High-Affinity K^+ Transporter1 (AtHKT1.1), which regulates shoot Na^+ by retrieval of Na^+ from the xylem into root xylem parenchyma cells.

KAT1: An inward-rectifying K^+ channel.

K^+/Na^+ ratio: The ratio of tissue or cytosolic K^+ concentration to Na^+ concentration. For technical reasons, it usually refers to the ratio in whole tissue, although the more biologically relevant ratio is that found in the cytosol. It is often used to show the ability of plants to exclude Na^+ under saline condition while maintaining uptake of K^+.

Leaf salt exclusion: The exclusion of Na^+ and Cl^- from the cytoplasm of leaves, the primary site of salt damage, under saline conditions.

Multigenic: Being due to more than one gene.

NHX: Na^+/H^+ exchanger, belonging to CPA1 family. NHX in arabidopsis has 6 members. AtNHX1-4 localize to tonoplast and sequester Na^+ and K^+ into vacuoles. AtNHX5 and 6 localize to Golgi and trans-Golgi network, and show higher affinity to K^+ compared to Na^+.

Osmotic adjustment: The adjustment of intracellular osmotic pressure, to enable maintenance of leaf expansion, leaf turgor and stomatal conductance as a means to minimize the toxic effects of the osmotic component of salt stress.

Rootstocks: Often refers to the underground part of a plant. In grafting, it refers to an already-established healthy plant root system, onto which a cutting or a bud from another plant can be grafted. The use of rootstocks is commonly for better fruiting and resistance to abiotic/biotic stresses of woody perennials such as grapevines and fruit trees.

ROS: Reactive oxygen species. Reactive chemicals containing oxygen (e.g. peroxides, superoxides, hydroxyl radical and singlet oxygen), induced by environmental stresses, which can cause damage to plant cells.

Saline/Salinity: High concentrations of NaCl in the soil (or hydroponic) solution.

SKOR: Stelar K^+ outward rectifier, a K^+ channel responsible for loading K^+ into the xylem apoplast from the stelar parenchyma cells.

Stele: The inner part of roots and stems of vascular plants, containing the xylem and phloem, providing the transport system between root and shoot. In roots, this is delimited by the endodermal cell layer.

Symplast: The volume inside the plasma membrane of cells, with the symplasm of neighboring cells connected by plasmodesmata. It allows the direct flow of water and small molecules (such as Cl^-) from the cytoplasm of one cell to another. In the root, symplastic flow refers to cell-to-cell movement through the plasmodesmata connecting cells of the epidermis, cortex and endodermis. Water and solutes moving by this path eventually reach the stele, for long-distance transport to the shoot.

Symport(er): A type of carrier transporter that is able to transport different solutes across a membrane in the same direction at the same time. It is a type of co-transporter, with a similar principle of action to an antiporter, except moving solutes in the same direction, rather than the opposite direction. Normally, the movement of one ion is down its difference in electrochemical potential, allowing the other ion to be moved against its difference in electrochemical potential.

Tissue tolerance: Tolerance of tissue, usually leaf, to accumulated Na^+ and/or Cl^-. This often refers to the cellular compartmentation of both ions into the vacuole, to protect the cytoplasm. It also refers to intracellular compartmentation of the both ions in epidermal cells of leaves to protect mesophyll cells, where most photosynthesis occurs.

Transporters: Transmembrane proteins that move solutes across a membrane. There are three types of membrane transporters (and transport classifications): channels (passive), carriers (secondarily active) and pumps (primary active). Channels moves solutes across membrane through its pore by selective diffusion. Carriers moves solutes across membrane by selective binding and significant conformational change. Pumps use energy, such as from the hydrolysis of ATP, to actively move ions such as H^+ or Ca^{2+} across membrane.

X-IRAC: Xylem-inwardly rectifying anion conductance – is low in occurrence and abundance, but up-regulated by ABA and Ca^{2+}.

X-QUAC: Xylem-quickly activating anion conductance – rapid activation and a transport capacity at physiological relevant membrane potentials sufficient to load all Cl^- and NO_3^- to the xylem; its activity is significantly inhibited by ABA and cytosolic Ca^{2+}.

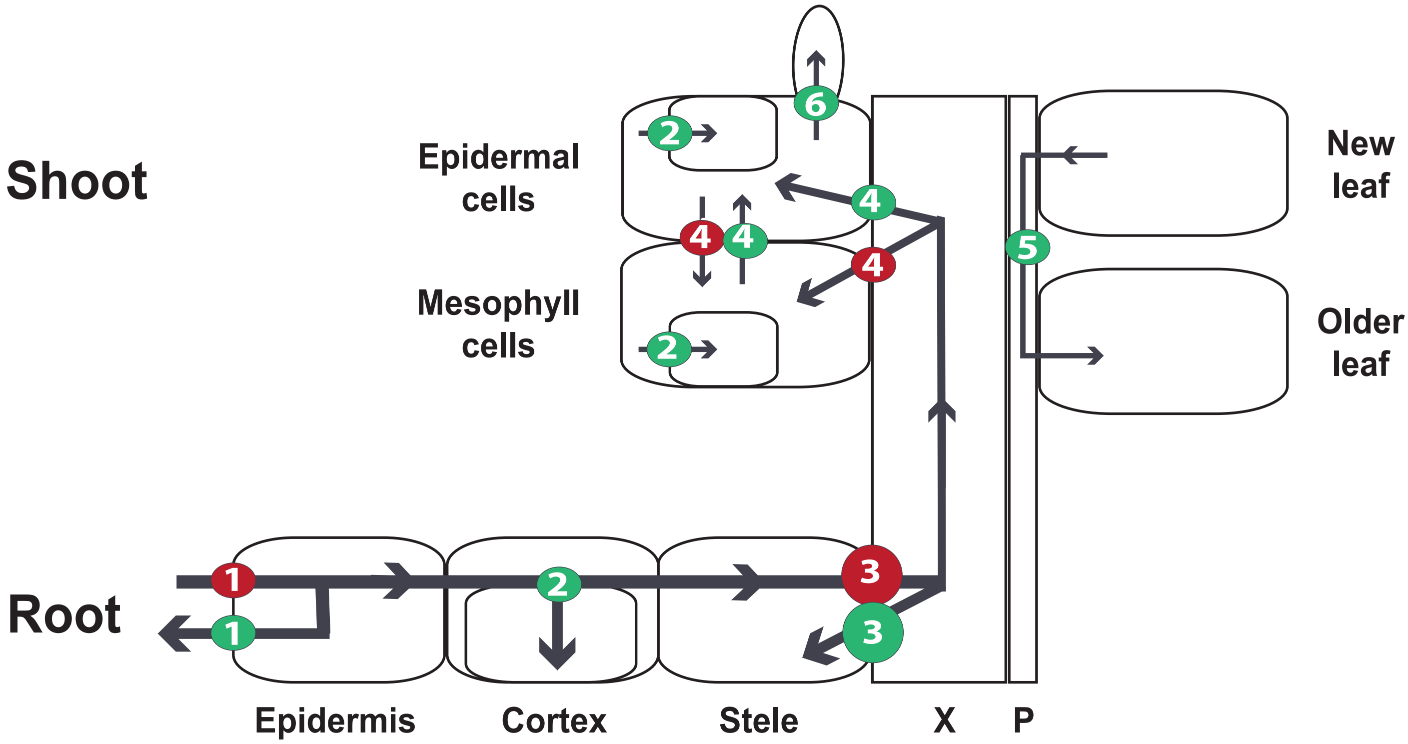
X-SLAC: Xylem-slowly activating anion conductance – is very low in transport capacity and has not been measured in maize stelar cells.

Figure legends

Fig 1 Mechanisms contributing to Cl^- exclusion from the leaf cytosol and thus Cl^- tolerance. Circles in green: processes that positively regulate Cl^- tolerance. Circles in red: processes that require inhibition to reduce cytosolic Cl^- load. X, xylem; P, phloem. In the root: (1) Minimizing net uptake across the root epidermis and cortex by increasing Cl^- efflux and decreasing its influx. Cl^- ordinarily enters root cells through secondarily active uptake whilst passive influx occurs at high salinities [102]. A salt-induced Cl^- efflux may exist to reduce the net uptake of Cl^- during salinity. (2) Maximizing intracellular compartmentation in vacuoles to reduce cytoplasmic Cl^-. This occurs in many cell types of plants; the root cortex may be a major location. Evidence has shown that the sequestration of Cl^- into root vacuoles can affect root-to-shoot long distance transport of Cl^-. (3) Minimizing net xylem loading (focus of the current review). This appears to be a major rate-limiting step for Cl^- exclusion from the shoot, and includes a reduction of passive loading and an increase of active retrieval. In the shoot: (4) Compartmentalizing Cl^- within leaf epidermis. This is to protect more important mesophyll cells, where photosynthesis occurs. (5) Maximizing phloem translocation from the newly expanded leaves to older leaves. Young leaves tend to be more sensitive to salt damage. Translocation of Cl^- from older leaves to younger ones could maintain/improve growth under salt stress. (6) Salt glands and bladders in halophytes. These are structurally specialized cells that can store or excrete Cl^- out onto the leaf surface. The excretion can be significant, but is unique to halophytes.

Fig 2 Thermodynamics of Cl^- transport between the xylem and surrounding cells. Likely differences in Cl^- activity, pH and membrane potential between the two compartments are indicated. Inside the cells of the root **symplast**, there is a higher concentration of Cl^- and a more negative membrane potential – both these favor the passive movement of Cl^- out of the cells into the xylem **apoplast**, down a difference in electrochemical potential. Cl^- retrieval can occur via coupling with the transport of H^+. This uses the difference in electrochemical potential for H^+ built up mainly by the H^+-ATPase, therefore this expends energy. Under salt stress. down-regulation of passive Cl^- loading and maximizing of retrieval are the processes that underpin the rate-limiting gatekeeper step in loading of Cl^- to the xylem (adapted from Fig 3b in [21]).

Fig 3 A model showing known and predicted Cl^- transporters affecting xylem Cl^- transport and Cl^- tolerance. The **symplastic** pathway for Cl^- in the root is highlighted in grey while the **apoplastic** pathway is highlighted in blue (dark blue in epidermis and cortex; light blue in stele). Candidates down-regulated by salt stress are highlighted in red, whereas those that are up-regulated are highlighted in green. This regulation can be transcriptional and/or posttranslational. Discussion and references for each candidate can be found in the manuscript. Also included are anion transporters that affect Cl^- exclusion in other cell types. In the shoot: AtCLCa [75, 91], AtCLCc [73], AtCLCg [74] and AtALMT9 [18]. In the root: AtNPF2.4 [14], AtSLAHs [12, 13], AtALMT9 [18], AtCLCc, CCCs [16, 45, 61, 62], AtNPF7.2 [50], AtNPF7.3 [49, 51] and GmSALT3 [15] and AtALMT12. Active Cl^- influx: Cl^- influx of root epidermal cells mediated by $Cl^-/2H^+$ symporters as described in [44]. Passive Cl^- influx: passive Cl^- influx of root epidermal cells when in saline conditions (membrane potential depolarized by Na^+ entry) as described in [102]. Passive Cl^- efflux of root epidermal cells favored by the electrochemical difference as reviewed in [2]. ALMT, Aluminium Activated Malate Transporter; CLC, Chloride Channel; CCC, Cation-Chloride Co-transporter; SLAH, SLAC1 Homolog; NPF, NRT1/PTR Protein Family; GmSALT3: salt tolerance-associated protein encoded on chromosome 3 (also referred to as GmCHX1/20). NAXTs: Nitrate excretion transporters.


Shoot
Epidermal cells
Mesophyll cells
New leaf
Older leaf
Root
Epidermis
Cortex
Stele
X
P
1
1
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2
2
3
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4
4
4
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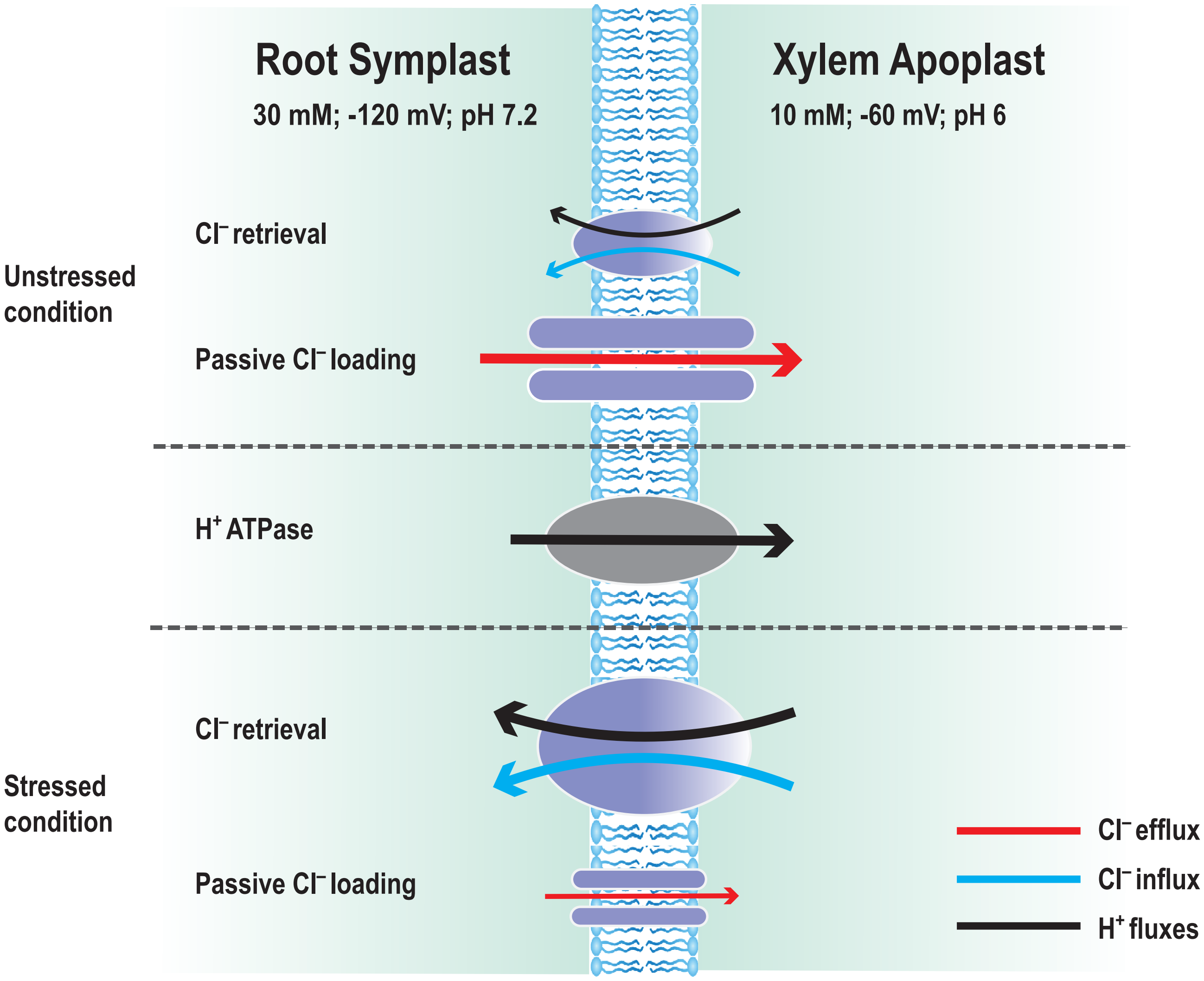
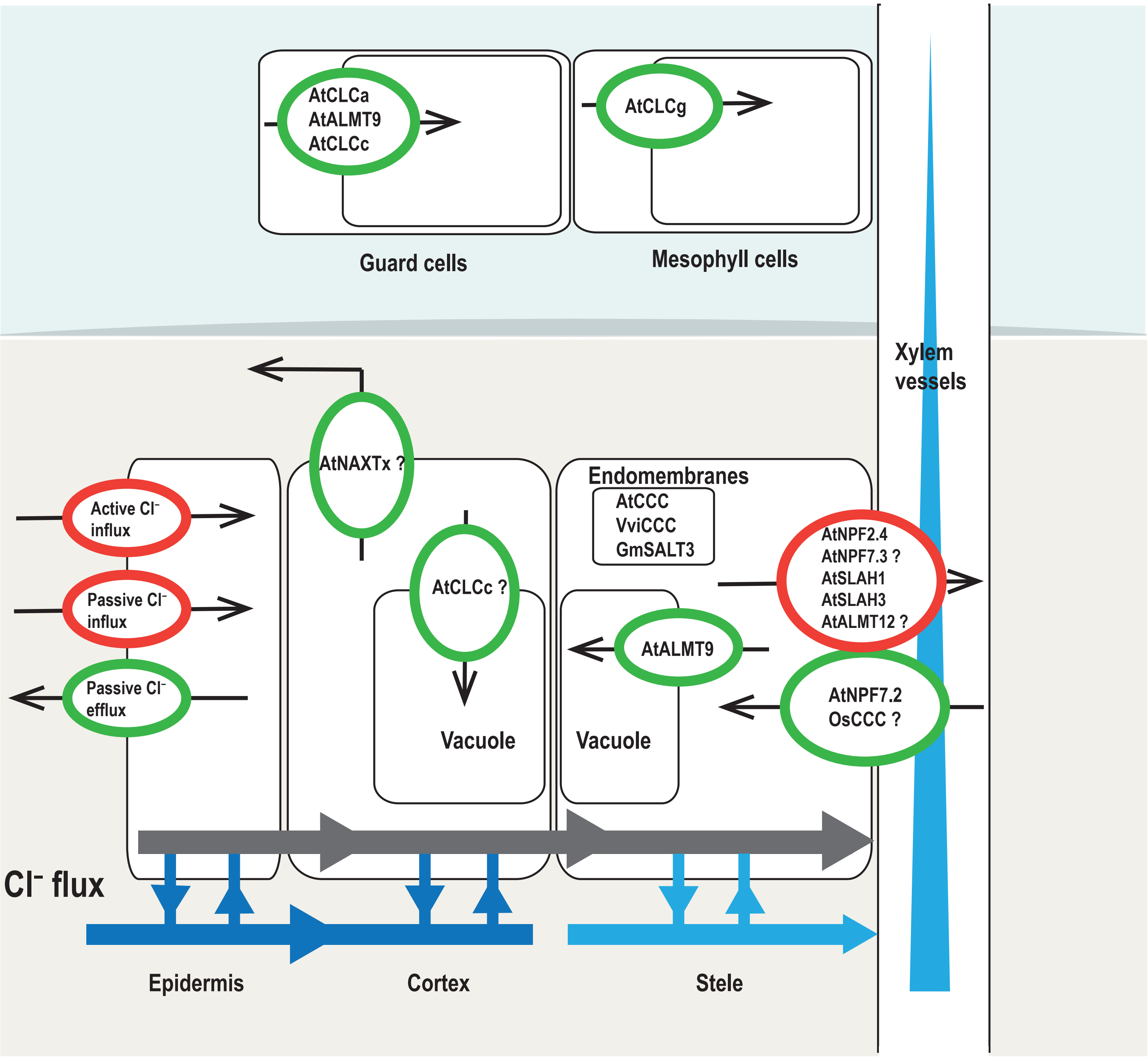

Root Symplast
30 mM; -120 mV; pH 7.2
Xylem Apoplast
10 mM; -60 mV; pH 6
Unstressed condition
Cl^- retrieval
Passive Cl^- loading
H^+ ATPase
Stressed condition
Cl^- retrieval
Passive Cl^- loading
Cl^- efflux
Cl^- influx
H^+ fluxes


AtCLCa
AtALMT9
AtCLCc
AtCLCg
Guard cells
Mesophyll cells
Xylem vessels
AtNAXTx ?
Endomembranes
AtCCC
VviCCC
GmSALT3
Active Cl⁻ influx
Passive Cl⁻ influx
Passive Cl⁻ efflux
AtCLCc ?
AtNPF2.4
AtNPF7.3 ?
AtSLAH1
AtSLAH3
AtALMT12 ?
AtALMT9
AtNPF7.2
OsCCC ?
Vacuole
Vacuole
Cl⁻ flux
Epidermis
Cortex
Stele