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Review

Anion channels in higher plants: functional characterization, molecular structure and physiological role

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Abstract

Anion channels are well documented in various tissues, cell types and membranes of algae and higher plants, and current evidence supports their central role in cell signaling, osmoregulation, plant nutrition and metabolism. It is the aim of this review to illustrate through a few selected examples the variety of anion channels operating in plant cells and some of their regulation properties and unique physiological functions. In contrast, information on the molecular structure of plant anion channels has only recently started to emerge. Only a few genes coding for putative plant anion channels from the large chloride channel (CLC) family have been isolated, and current molecular data on these plant CLCs are presented and discussed. A major challenge remains to identify the genes encoding the various anion channels described so far in plant cells. Future prospects along this line are briefly outlined, as well as recent advances based on the use of knockout mutants in the model plant *Arabidopsis thaliana* to explore the physiological functions of anion channels in planta.

Keywords: Anion channel; CLC; Plant; Plasma membrane; Tonoplast

1. Introduction

In higher plants, vital processes, such as mineral nutrition, carbon and nitrogen metabolism, and more generally growth and development strongly depend on solute and water fluxes across the cell plasma membrane, tonoplast and other endomembranes. Among the various transport systems involved in these basic cellular functions, ion channels represent a large class with highly diversified properties. These proteins facilitate passive fluxes of ions down their respective electrochemical gradients. In plant cells as in animal cells, ion channels are thought to fulfil three main physiological functions: cell osmoregulation because of their ability to accommodate over short periods large net ion fluxes, cell signaling by amplification and propagation of electrical signals or transport of secondary messengers, such as Ca^{2+}, and control of the membrane potential. Owing to

Abbreviations: ABA, abscisic acid; 9-AC, anthracene-9-carboxylic acid; CLC, member of the family of voltage-dependent chloride channels; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; GCAC1, guard cell anion channel 1; GFP, green fluorescent protein; IAA-94, *R*(+)-methylindazone; indanyloxyacetic acid 94; NA, niflumic acid; NPPB, 5-nitro-2,3-phenylpropylaminobenzoic acid; SITS, 4-acetamido-4′-isothiocyanato-stilbene-2-2′-disulfonic acid

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the use of the patch-clamp technique [1], the knowledge on plant ion channels has been rapidly growing over the last 15 years. Studies on different plant species and various cell types have revealed that all subcellular membranes investigated so far (plasma membrane, tonoplast, plastidial and mitochondrial membranes) are equipped with a variety of channels exhibiting different ion selectivities and specific regulation mechanisms [2–5].

In contrast to animal cells, plant cells have evolved unique electrical properties, mostly based on the transport of H^+, K^+ and anions. In plants, the highly negative plasma membrane potential of resting cells (−100 to −200 mV) is primarily determined by the H^+-ATPase which extrudes protons into the apoplast. Because of the low abundance of extracellular cations as counterions for H^+ transport, anion channels allowing anion efflux across the plasma membrane can play a critical role in the down regulation of the membrane potential. In addition, besides chloride, plants need to transport various anions, such as nitrate, sulfate, phosphate, and organic acids for mineral nutrition and metabolism. Finally, the high compartmentation of plant cells in cytosol, plastids, mitochondria and large central vacuole suggests the existence of a great variety of transport systems for anions. Many anion channel activities have been indeed documented in various tissues, cell types and membranes of algae and higher plants [6,7]. In contrast, information on their molecular structure has only started to emerge. It is the aim of this review to illustrate through a few selected examples the variety of anion channels operating in plant cells and some of their regulation properties and unique physiological functions. Novel properties revealed by current functional and molecular data on these channels will be discussed.

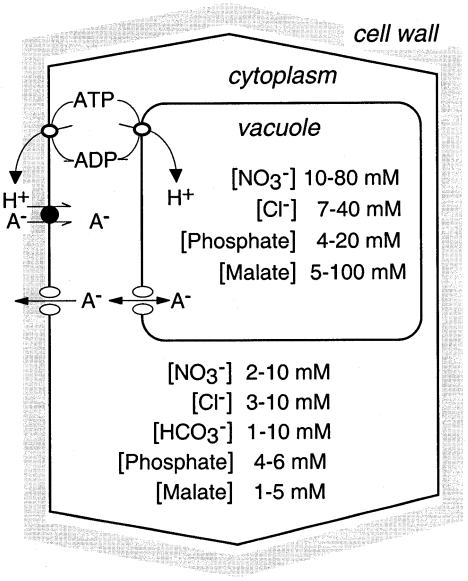


Fig. 1. Anion distribution in plant cytoplasmic and vacuolar compartments. H^+-ATPases generate proton electromotive forces across the plasma membrane and the vacuolar membrane. Anions are actively taken up into the cytoplasm by anion/proton symport systems operating at the plasma membrane. The negative plasma membrane potential together with the anion concentration gradient drive passive fluxes of anions out of the cell through plasma membrane anion channels. The high anion concentration in the vacuole may result from passive anion fluxes driven by the negatively charged tonoplast, through vacuolar anion channels.

2. Anion compartmentation in plant cells

The high turgor generally displayed by walled plant cells is generated by a high intracellular osmolarity, in the range of 300–500 mOsm, which comes in part from the accumulation of soluble potassium salts. Organic and/or inorganic anions provide the electroneutrality, but their relative concentrations fluctuate depending on physiological and environmental parameters. Fig. 1 illustrates that in plant cells, the highest anion concentrations are found in the vacuole which represents a storage compartment while cytosolic levels are maintained in the millimolar range. Inorganic anions are usually of low abundance in the soil solution, and more generally the apoplastic compartment (the cell wall network) represents only a small anion reservoir because of its reduced volume and the negative charges of the cell wall. To account for the observed concentrations in plant cells (Fig. 1), anions must be actively taken up by anion/proton symport systems operating at the plasma membrane and described in numerous plant species [8,9]. Conversely, the membrane electrical polarization, together with the typical anion compartmentation observed in most plant cells will drive passive fluxes of anions out of the cell through plasma membrane anion channels. A salt release following from such an opening of plasma membrane anion channels may cause transiently a high local

concentration in the cell wall. The high vacuolar concentration may result from passive fluxes driven by the negatively charged tonoplast, through channels on the vacuolar membrane.

The major mineral anions in plant tissues are nitrate, chloride, sulfate and phosphate. Carbonate, despite its low concentration compared with other inorganic anions, occupies a particular status because of its role in intracellular pH regulation and because it is the major carbon input for photosynthesis. Due to its primary role in nitrogen assimilation, transport and cell compartmentation of nitrate have been particularly well studied in the root [8,10]. In plants supplied with non-limiting nitrate, the concentration of this anion can be 5–20 times higher in the vacuole than in the cytosol [11–13]. Van der Leij et al. [13] showed, for instance that, in barley root cells, cytosolic nitrate is maintained fairly constant at around 4 mM whatever the growing conditions, while vacuolar nitrate can be readily accumulated or mobilized as a function of nitrogen demand. Measurements of the inorganic phosphate content in pea or maize roots by the ^{31}P-NMR technique also showed that the cytoplasmic phosphate content is kept constant at 4–6 mM over a wide range of phosphorus nutrition, whereas the vacuolar content fluctuates from 4 to 20 mM in the roots of P-sufficient plants down to unmeasurable too low values upon P-starvation [14]. Despite the lack of a complete compartmental analysis of other anions, a few thorough studies on the anion content of plant tissues provide estimations of intracellular anion concentrations. With the exception of halophytes, vascular plants have an internal chloride concentration of several tens millimolar (5–133 mM for different species as reviewed by [15]). Cell sap and global analyses of sulfate content give a large range of values from 1 to 92 mM [15,16], depending on the ionic status. These values probably reflect concentrations in the vacuole since chloride and sulfate have been shown to be mainly stored in this compartment [17]. In *Lemna minor*, for instance, about 70% of total sulfate is bound within cellular organic thio-compounds, the 30% free sulfate being distributed between the vacuole (25%), the cytoplasm (1%) and the cell wall (1%) [18].

Organic acids represent a variable fraction of total soluble anions, and increase when the inorganic anions are insufficient in the external medium [19,20]. They primarily derive from cell metabolism and are rarely taken up from the external medium because of their low availability in the soil. However, cell-to-cell transfer of organic acids has been described in plant tissues [21] and this results in transient pools of organic anions in the apoplast. Malate is the most abundant organic acid in plant cells. The analysis of plant tissues revealed fluctuations of malate concentrations depending on organs, plant species [22] and environmental conditions. Malate plays a major role in the osmotic regulation of plant cells. In *Vicia faba* guard cells, malate is accumulated during stomatal opening [23,24] from 38 mM in the closed state to 75 mM in the open state, or even to 145 mM when there is no chloride in the external medium, indicating that malate palliates the chloride deficiency. A different role has been described for malic acid in crassulacean acid metabolism (CAM) plants where it serves as a carbon storage source for photosynthesis: during the night, the malate pool increases, as a result of CO_2 assimilation, while during the day CO_2 is mobilized and used in the photosynthesis pathway. The cytosolic concentration of malate is low, ranging from 1 to 5 mM. Thus, most of the cellular malate is stored in the vacuole as demonstrated in *Catharanthus roseus*, a C3 plant [25] or in *Bryophyllum daigremontanium*, a CAM plant [26]. Other organic acids, such as citrate, are accumulated in a similar ratio within the vacuole in *Hevea brasiliensis* [27].

3. Functional characteristics, regulation and cellular functions of plant anion channels

Most of information available on plant anion channels has been obtained at the plasma membrane level. It was mentioned above that, because of the highly negative transmembrane potential and outward-directed anion gradients across this membrane (Fig. 2), the opening of anion channels results in anion release from the cytoplasm to the extracellular space. Anion channel activation will thus induce membrane depolarization, which in turn may activate other voltage-dependent channels, such as Ca^{2+} channels, or serve as an electrical shunt for the H^+-ATPase which extrudes H^+ ions into the

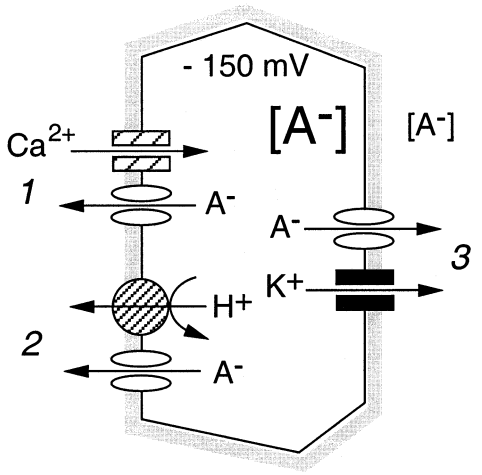


Fig. 2. Major cellular functions of plasma membrane anion channels. Because of the highly negative transmembrane potential and outward-directed anion gradients across the plasma membrane, the opening of anion channels results in anion release from the cytoplasm to the extracellular space. Anion channel activity is coupled with the activity of other transporters, such as Ca^{2+} channels, H^+-ATPase or K^+ channels and contributes to three major functions: 1, electrical signaling and calcium signaling; 2, control of membrane potential and pH gradients; 3, osmoregulation. See text for further comments.

apoplast. These aspects may contribute to short-term electrical and/or calcium signaling, and to the regulation of membrane potential and pH gradient across the plasma membrane. Membrane depolarization may also activate outwardly directed channels mediating potassium efflux and lead to a net salt loss, further driving water movements and participating in cell osmoregulation. All these processes have to be prevented in cells at rest where a tight regulation of anion channel activity is expected. The following examples, illustrated in Fig. 3, will provide an overview of the basic characteristics of plant anion channels, in terms of transport activity, regulation and physiological functions.

3.1. Cell signaling and osmoregulation: the stomatal model

The aerial parts of plants exchange carbon dioxide and water with their environment through small pores called stomata, whose aperture is tightly regulated by two guard cells. A variety of environmental (light, CO_2) and physiological (hormones) stimuli influences the opening/closing movement of stomata, and the contribution of guard cell ion channels to these phenomena has been extensively studied [3,28–30]. The opening of the stomatal pore follows from an increase of guard cell turgor caused by an accumulation of salts and an accompanying osmotic water influx. Chloride and malate represent the major anionic species involved and balance the K^+ charge. Chloride (and potassium) accumulation is mediated by various transport systems (transporters and ion channels) and is driven by the high electrochemical proton gradient built up by the plasma

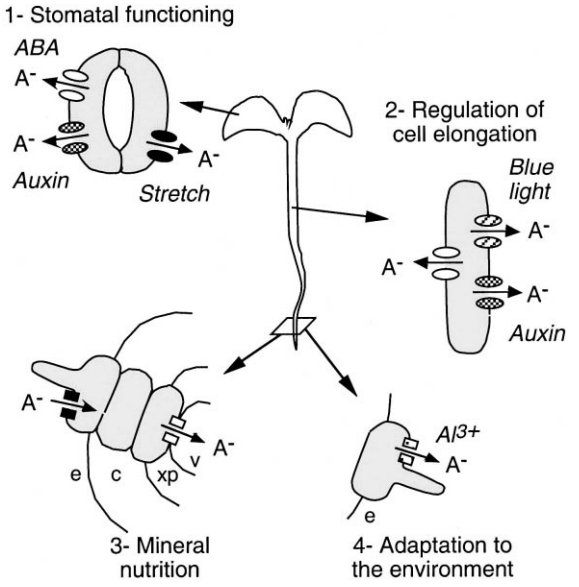


Fig. 3. Some examples of the physiological roles of plasma membrane anion channels in various plant cells and tissues. Putative endogenous or environmental regulators of channel activity are indicated in italics. (1) Stomatal functioning: stomatal guard cells are equipped with at least three anion channels which contribute to signaling and osmoregulation processes underlying the opening/closing movements of stomata. (2) Regulation of cell elongation: three anion channels co-exist in hypocotyl cells and mediate signaling processes involved in the control of cell elongation by hormones and light. (3) Mineral nutrition: in the root, anion channels of epidermal cells may participate in the anion uptake from the soil solution, but only in certain circumstances (ample supply of nitrate, salt stress). Xylem parenchymal cells possess channels allowing the release of anions into xylem vessels as the first step of their translocation to the shoot (e, epidermis; c, cortex; xp, xylem parenchyma; v, vessel). (4) Adaptation to the environment: an anion channel activated by aluminum is present in roots from cereal plants and could be responsible for the onset of aluminum tolerance in certain wheat cultivars.

membrane H^+-ATPase, while malate is synthesized from plastidial starch in response to stimuli inducing stomatal opening. Conversely, the pathway for ion efflux leading to stomatal closure is provided by voltage-dependent anion channels together with outward-rectifying potassium channels [3,31]. Much effort has been devoted to analyzing the stomatal closing response induced by the stress hormone abscisic acid (ABA) (reviewed in [32]).

Patch-clamp studies on guard cell protoplasts from *Vicia faba* have revealed the presence of at least three types of plasma membrane anion channels: two voltage-dependent channels and a mechanosensitive channel [33–35] (Table 1, Fig. 3). The voltage-dependent channels are activated by membrane depolarization, but with different kinetics. One of them, called GCAC1, shows fast activation and deactivation kinetics in the 10-ms range and was also termed 'R-type' (for rapid-type) channel [33,36]. The second shows slow kinetics in the 10-s range and was termed 'S-type' (for slow-type) channel [35,37]. A slow anion channel is also present in guard cells from *Arabidopsis thaliana* ([38], Table 1). A striking feature common to the R-type and S-type anion channels from guard cells is their multiple levels of regulation, from both external (apoplastic) and internal (cytosolic) sides of the plasma membrane (reviewed in [31,39]) (Table 1). Of particular interest is their modulation by calcium [40,41], external anions [36,42–44] and cytosolic ATP [40,45,46], and their sensitivity to plant hormones, such as auxin [44,47] and abscisic acid [38,48], which provides obvious links to guard cell signaling cascades.

The question whether anion channels are involved in stomatal movements was primarily investigated using a pharmacological approach (Table 1), and indeed measurements on epidermal peels from *V. faba* and *Commelina communis* showed that stomatal responses can be modulated by anion channel blockers [49]. In particular, inhibition of stomatal opening by ABA was alleviated by the presence of 9-AC, probenecid or niflumic acid. The idea that anion channels would participate in stomatal closing is also supported by data from Schroeder et al. [50] who showed that exogenous application of another anion channel blocker, NPPB, completely abolishes stomatal closing triggered by a combined exposure of guard cells to ABA and malate. In this latter case, DIDS does not modify significantly the ABA response. This pharmacological profile, in particular the insensitivity to DIDS, closely match the inhibition profile of the slow anion channel present in the guard cell plasma membrane [49–51], but differs from the profile of the R-type channel [52,53]. Although the effects of anion channel blockers should be interpreted with caution, as some of them appeared to be potent inhibitors of plant K^+ outward rectifying channels [54], these pharmacological data, together with data showing a regulation of the slow channel by ABA and protein phosphorylation processes [45] (Table 1), provide converging evidence for the involvement of this channel in ABA-induced stomatal closure. This was recently confirmed, in *A. thaliana*, by the electrophysiological analysis of *abi1* and *abi2* (*abscisic acid-insensitive 1* and *2*), two phosphatase 2C mutants disrupted in their ABA response [32]. The *abi1* and *abi2* mutants are insensitive to ABA for stomatal closure, and ABA fails to activate the S-type channel in these plants [38]. A novel step in the ABA response pathway was uncovered by the study of *era1* (*enhanced response to ABA*), a mutant altered in a farnesyltransferase gene [55]. This mutant shows a hypersensitivity to ABA, a more pronounced stomatal closing, and an overactivation of the slow anion channel. These phenotypic traits can be mimicked by application of HFPA (α-hydroxyfarnesylphosphonic acid), a farnesyl transferase inhibitor [56]. The most recent discovery in tobacco of a syntaxin (Nt-Syr1) that is associated with the plasma membrane, and is implicated in the anion channel response to ABA in guard cells, adds a new element in the ABA/channel signaling cascade [57]. All these studies reinforce the idea that S-type anion channels play a central role in controlling stomatal closure in response to ABA [3], and at the same time uncover novel mechanisms for channel regulation.

In contrast, the physiological function of the R-type anion channel in guard cells is not as clearly established. On the one hand, its modulation by auxin, which was also observed on a rapid-type anion channel from tobacco cell suspensions [58], may indicate a role in hormone signaling. The fact that the stomatal aperture induced by auxin exhibits a bell-shaped dose–response curve [47], and that the channel responds only to supra-optimal auxin concentrations suggests that it would rather contribute to re-

Table 1
Basic characteristics of some plasma membrane and tonoplast anion channels from various plant cell types as revealed by patch-clamp studies

Cell type (plant species)	Membrane	Voltage dependence	Kinetics	Selectivity	Regulation pattern	Pharmacology	Physiological function	References
Guard cell (*Vicia faba*)	plasma membrane	strong activated by depolarization	rapid (ms range)	$NO_3 > I > Br > Cl$ > malate	activated by int. ATP (nucleotide binding) and Ca^{2+}; regulated by auxin, pH, ext. anions, CO_2	DIDS > NPPB, IAA94 > 9-AC	hormone signaling excitability?	[31,33,36,39, 40,42–44,46, 47,52,53]
Guard cell (*Vicia faba*)	plasma membrane	weak activated by depolarization	slow (s range)	$NO_3 > Br > F$ > $Cl > I >$ malate	activated by int. ATP (phosphorylation) and Ca^{2+}; regulated by ABA;	NPPB, IAA-94 > 9-AC ≫ DIDS	osmoregulation (stomatal closing)	[31,35,37,41,45, 48–51]
Guard cell (*Arabidopsis thaliana*)	plasma membrane	weak activated by depolarization	slow (s range)	Cl	activated by int. ATP (phosphorylation) and ABA	9-AC ≫ NPPB, NA, DIDS	osmoregulation (stomatal closing)	[38,51]
Guard cell (*Vicia faba*)	plasma membrane	weak or no voltage depend.	n.d.	Cl	activated by membrane stretch	n.d.	regulation of volume and turgor of guard cells	[34]
Guard cell (*Vicia faba*)	tonoplast		n.d.	Cl, malate	activated by Ca-dependent protein kinase and ATP	NA	osmoregulation (stomatal opening)?	[61]
Hypocotyl epidermal cells (*Arabidopsis thaliana*)	plasma membrane	strong activated by depolarization	rapid (ms range)	$NO_3 \geq SO_4 > Cl$ > $HCO_3 >$ malate, glutamate	inhibited by int. ATP (nucleotide binding)	NA, NPPB > IAA94 ≫ 9-AC, DIDS	electrical signaling?	[65,66]
Hypocotyl epidermal cells (*Arabidopsis thaliana*)	plasma membrane	weak activated by depolarization	slow (s range)	$NO_3 > Cl \gg SO_4$	activated by int. ATP	DIDS > SITS, NA, NPPB ≫ IAA-94, 9-AC	auxin-induced growth inhibition?	Frachisse, unpublished results
Hypocotyl cells (*Arabidopsis thaliana*)	plasma membrane	weak	n.d.	Cl	activated by blue light; Ca^{2+}-dependent	NPPB	blue light-induced growth inhibition	[70,71]
Root cells (*Triticum aestivum*)	plasma membrane	activated by depolarization	rapid (ms range)	$NO_3 > Cl > I$ ≫ H_2PO_4	activated by int. Ca^{2+} and ext. anions	DIDS ≫ Zn	anion uptake under high salinity?	[75]
Root cells (*Hordeum vulgare*)	plasma membrane	n.d.	slow	Cl	n.d.	n.d.	salt release from xylem parenchyma?	[77]
Root cells (*Triticum aestivum*)	plasma membrane	activated by hyperpolarization	n.d.	Cl	regulated by ext. anions; activated by ext. Al^{3+}; insensitive to La^{3+}	NA	aluminum tolerance?	[80]

n.d., not determined.

ducing stomatal opening. On the other hand, the voltage-dependence and kinetics of the rapid anion channel are reminiscent of ion channels in excitable membranes [59]. This raises the idea that this rapid anion channel could mediate transient changes in membrane potential and be responsible for guard cell excitability [39,60].

Until recently, there was no data available on tonoplast channels which could account for the the large flow of anions across the tonoplast, involved in stomatal closing or opening. Pei et al. [61] reported that a calmodulin-like domain protein kinase (CDPK) from *A. thaliana*, and to a lesser extent animal protein kinase A, can activate novel currents in the guard cell vacuolar membrane of *V. faba* (Table 1). These currents are carried by chloride or malate, and their activation by CDPK is dependent on both cytosolic Ca^{2+} and ATP. These vacuolar chloride (VCL) channels activate mainly at negative physiological potentials on the cytosolic membrane side and likely provide a pathway for Cl^- uptake during stomatal opening.

3.2. Regulation of cell elongation in the hypocotyl

Cell expansion in plant tissues is regulated at the organ level by endogenous signals (growth hormones) as well as by environmental factors such as light, temperature, gravity and water availability. It involves the coordinated control of cell wall synthesis and relaxation, solute and water transport, and membrane biogenesis [62]. The hypocotyl of dicotyledonous seedlings has been widely used as a model system for studying the mechanisms and regulation of these processes. In particular, the cellular basis of growth has been thoroughly described in the hypocotyl of *A. thaliana* [63] and a number of mutants with altered hypocotyl growth are available in this species.

Several plant hormones exert on the long term (several days) stimulatory or inhibitory effects on hypocotyl growth. When exogenously applied to intact *Arabidopsis* seedlings, auxins inhibit hypocotyl cell elongation. Thomine et al. [64] used a pharmacological approach to investigate the possible contribution of anion channels to this process. Three anion-channel blockers, 9-AC, DIDS and SITS, which produced no or little effect on the hypocotyl elongation by themselves, were able to counteract the auxin-induced growth inhibition. The observed effects of auxin and anion channel inhibitors on overall hypocotyl length were paralleled by their effects on epidermal cell size. This hormone/blocker interaction appeared to be specific for auxins since it did not occur when hypocotyl elongation was inhibited by other growth regulators, such as ethylene or cytokinins [64]. These data suggest a contribution of anion channels in the regulation of *Arabidopsis* hypocotyl growth by auxin (Fig. 3). Parallel electrophysiological investigations showed that hypocotyl epidermal cells from young *Arabidopsis* light-grown seedlings are equipped with a plasma membrane anion channel [65] which shares similar properties with the R-type channel from guard cells (Table 1). This channel is tightly controlled by transmembrane voltage, with activation and deactivation kinetics in the ms range. Voltage regulation is under the control of cytosolic nucleotides and likely involves a nucleotide binding site [66]. More recently, our group identified a second anion channel resembling the S-type channel from guard cells and co-residing with the rapid anion channel at the plasma membrane of hypocotyl cells (Frachisse et al., unpublished results) (Table 1). This channel is also regulated by intracellular nucleotides. Further work is obviously needed to establish the physiological role of the rapid- and slow-type anion channels in the hypocotyl (Fig. 3). Based on their kinetic parameters, it can be hypothesized that the rapid channel could contribute to electrical signaling in hypocotyl cells, while the slow channel could be involved in massive anion effluxes. The efficiency of DIDS to block the slow channel activity and to counteract the auxin effects on growth, together with the insensitivity of the rapid channel to this blocker, favors the idea that the slow anion channel could contribute to the down regulation of hypocotyl cell elongation by auxin.

Light exerts its effects through the combined action of several photoreceptors which sense the quality and intensity of the light signal [67]. Seedlings of several dicotyledonous species respond to a blue light (BL) treatment by a large transient depolarization of the plasma membrane followed by a rapid inhibition of stem elongation [68–70]. In etiolated cucumber hypocotyls, the mechanism responsible for the BL-induced depolarization was shown to involve H^+-

ATPase inhibition and probably anion channel activation [69]. Patch-clamping, in the cell-attached mode, of protoplasts from etiolated *A. thaliana* hypocotyls showed that blue light also activates a calcium-dependent plasma membrane anion channel in this material [70,71] (Table 1). The channel is sensitive to the anion channel blocker NPPB, which also inhibits the depolarization induced by BL in intact seedlings and reduces the inhibitory effects of BL on hypocotyl growth. It was inferred from these data that activation of the anion channel plays a role in transducing the BL signal into growth inhibition (Fig. 3). By comparing BL responses in *Arabidopsis* seedlings from wild-type and the *hy4* (*hypocotyl4*) mutant lacking the BL receptor, Parks et al. [72] could separate two phases in the BL-induced growth inhibition. They show that anion channel activation does not correlate with the rapid inhibition phase, but rather contributes to the long-term growth response mediated by the HY4 BL receptor. The response to BL has also been investigated in protoplasts isolated from maize coleoptiles or *Arabidopsis* hypocotyls. These protoplasts shrink transiently in response to a pulse or continuous treatment with BL [73,74]. In both cases, BL-induced protoplast shrinking can be blocked by NPPB, suggesting once more that anion channel activation would constitute an early step following BL perception. However, the question of how this early membrane response contributes to the integrated cell expansion response at the organ level still needs to be addressed. Another open question concerns the possible correspondence of the blue-light activated anion channel observed in dark-grown hypocotyls [70] with the two voltage-dependent anion channels present in light-grown hypocotyls ([65], Frachisse et al., unpublished results).

3.3. Mineral nutrition and adaptation to the environment in root cells

Anion channels are less documented in root cells compared to stem cells, but a number of studies in cereals highlights their putative functions in nutrient transport and tolerance to soil metals (Fig. 3). Skerrett and Tyerman [75] described in wheat root protoplasts an anion channel that allows inwardly-directed fluxes of Cl^- and NO_3^- at depolarized membrane potentials or at high external anion concentration (Table 1). The anion currents are activated by intracellular Ca^{2+} and enhanced by external anions. DIDS, which was previously shown to reduce Cl^- uptake in corn root protoplasts [76], partially blocks the anion channel. These features led the authors to propose that this channel may be important for membrane potential regulation, and can play a significant role for anion uptake, but only when the membrane potential becomes more positive than the equilibrium potentials for permeant anions. These particular conditions may be encountered when the root faces an ample supply of NO_3^- or a salinity (NaCl) stress (Fig. 3). After mineral ions have been taken up by the root, they have to be transported toward the shoot. In agreement with this, Wegner and Raschke [77] identified in barley roots a slow-type channel allowing anion efflux from root xylem parenchyma cells (Table 1). This anion channel would need further characterization, but it is postulated to participate, together with potassium channels, in salt release into the xylem sap for subsequent translocation to the shoot (Fig. 3).

Aluminum (Al^{3+}), a prevalent cation in soils, becomes toxic for plants upon dissociation in acidic conditions and can inhibit root growth at micromolar concentrations. A comparative study of Al^{3+}-sensitive and Al^{3+}-tolerant wheat cultivars revealed that Al^{3+}-tolerant cultivars extrude higher levels of malate into the cell wall space than the Al^{3+}-sensitive cultivars [78]. Thus, aluminum tolerance might be based on the secretion of malate, which functions as a potent chelator and detoxifier of Al^{3+}. Further characterization of Al^{3+}-stimulated efflux of malate from Al^{3+}-tolerant wheat roots, in particular its sensitivity to various anion channel blockers, led to the suggestion that malate efflux was occurring via anion channels in apical root cells [79]. In agreement with this, Ryan et al. [80] discovered, in protoplasts isolated from the apex of Al^{3+}-tolerant wheat roots, a novel channel which is activated by Al^{3+} and allows Cl^- efflux (Table 1). Several properties of this channel are similar to those of the Al^{3+}-activated efflux of malate from the roots of tolerant plants, but its permeability to malate remains to be demonstrated. In addition, the channel was observed in protoplasts from both Al^{3+}-sensitive and Al^{3+}-tolerant plants. This does not fit at first sight with the idea that

this channel would be responsible for the onset of aluminum tolerance, but transduction events leading to channel activation by Al^{3+} may still be different in the root of tolerant and sensitive cultivars.

3.4. Long-distance signaling mediated by action potentials

The variations in transmembrane potential which are propagated from cell to cell and enable long-distance signaling have been mostly designated as action potentials (AP). Even though AP have been studied to a much lesser extent in plants than in animals, AP have been found in almost all organisms investigated, from fungi and algae to higher plants [81–87]. The sequence of events occurring during the plant action potential was elucidated primarily in the giant internodal cells of the green alga *Chara corallina* [88]. In this cellular model, AP are initiated by the opening of voltage-dependent Ca^{2+} channels in the plasma membrane and in the tonoplast [86,89]. The resulting elevation of free cytoplasmic Ca^{2+} concentration causes the opening of Ca^{2+}-dependent anion channels, which leads to a plasma membrane depolarization [90,91]. This depolarization in turn activates K^+-outward delayed rectifier channels which mediate a strong K^+ efflux and allow membrane repolarization towards the resting potential [92,93].

Motile higher plants such as *Mimosa pudica* typically develop action potentials to trigger leaf movements in response to an external stimulation [84]. Action potentials have also been described in non-motile plants, and take place, for instance, in response to a mechanical damage in hypocotyls of young dicot plants such as *Bidens pilosa* [94] or in cotyledons of tomato seedlings [95]. Similarly, oscillations in free-running voltage have been recorded at the plasma membrane of *V. faba* guard cells [60]. Only a few studies have addressed the function and conduction pathways of AP in higher plants [94–98], but studies conducted on *M. pudica* have suggested the involvement of chloride channels as observed in *Chara*. Interestingly, the rapid voltage-dependent anion channels described in *V. faba* guard cells [33,47] and in *A. thaliana* hypocotyl cells [65] display N-shape current–voltage curves reminiscent of Na^+ channels in animal nerve or muscle cells, and more generally of voltage-dependent channels typical from excitable cells [59]. This suggests that, instead of using Na^+ channels, plant cells take advantage of voltage-dependent anion channels to depolarize the plasma membrane and propagate electrical signals.

3.5. Tonoplast anion channels as regulators of cell metabolism

With a storage capacity of more than 90% of the anions contained in the plant cell, the vacuole plays a central role in cell homeostasis and metabolism [99]. In particular, the vacuolar membrane (or tonoplast) controls the availability in malate, nitrate and phosphate which can be metabolized in the cytoplasm and the plastids. The patch-clamp technique, which can be easily applied to isolated plant vacuoles, has permitted the identification of several distinct types of tonoplast anion channels. At low cytoplasmic Ca^{2+} concentration, the tonoplast ion conductance is dominated by instantaneously activated fast-vacuolar (FV-type) channels, with a low selectivity for anions [100]. In contrast, slow-vacuolar (SV-type) channels account for most of the ion conductances at high cytoplasmic Ca^{2+} concentration. This channel is strongly voltage-dependent and was claimed to have similar selectivity for cations and anions [100,101], but this latter feature is still controversial [61]. More recently, Dunlop and Phung [102] provided some evidence in red beet for a phosphate permeable tonoplast channel that contributes to the slow vacuolar current. This set of data likely reflects the fact that several kinds of channels can contribute to the SV whole vacuolar current. In sugar beet vacuoles, Plant et al. [103] described a chloride-activated inward anion channel, whose permeability sequence (chloride > malate > acetate > nitrate > phosphate) is shifted to (acetate > nitrate > phosphate > chloride > malate) by an increase in vacuolar chloride concentration. The activation of this channel by vacuolar chloride would then provide a pathway for the storage of nutrients, such as nitrate and phosphate in the vacuole, while the reduction in malate current would favor the use of malate in respiration and cytoplasmic pH control.

Malate is actually a ubiquitous anion which plays an important role in carbon metabolism and as a charge-balancing anion in the vacuole. In CAM plants, malate is produced as a result of dark CO_2

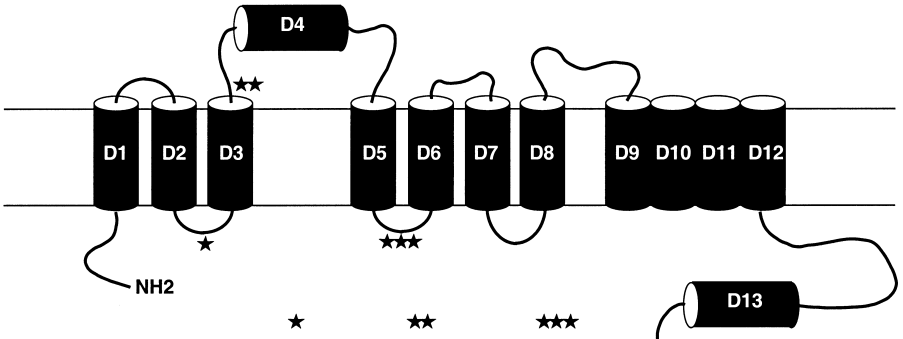
fixation and is stored in the vacuole, from which it can be remobilized during the following light period. Two anion channels, one favoring malate uptake and the other malate efflux have been identified by Iwasaki et al. [104] on the tonoplast of *Graptopetalum*. These channels could provide a mechanism for homeostasis and diurnal rhythm in leaf cells of this CAM plant. In the tonoplast of mesophyll cells from *Kalanchoe daigremontiana*, another CAM plant, the membrane conductance is dominated by a vacuolar malate channel or VMAL [105] which shows strong rectification, slow activation kinetics and lack of Ca^{2+} dependence. In vacuoles from sugar beet and *Arabidopsis* cell suspensions, currents corresponding to $malate^{2-}$ or $succinate^{2-}$ entry in the vacuole have also been recorded [106,107]. All these studies clearly show that VMAL channels provide a major route for malate uptake into the vacuole of both C3 and CAM plants.

4. Molecular structure of plant anion channels

Although the functional characteristics and physiological roles of plant anion channels have been established in detail in some cases, little is known about the molecular structure of these channels. Up to now, biochemical approaches and expression cloning in heterologous systems have been unsuccessful in isolating any anion channel gene. By contrast, the search for homologs of animal anion channels has enabled the cloning, in tobacco [108] and *Arabidopsis* [109], of members of the family of voltage-dependent chloride channels (CLC) (reviewed in [110]).

4.1. The plant CLC family

The first member of the CLC family, called *CLC-0*, was isolated from *Torpedo marmorata* electric organ by expression cloning in *Xenopus* oocytes [111]. The CLC family comprises members in bacteria [112], yeast [113] and mammals [110]. Nine different *CLC* genes are known at present in mammalian cells. They display differential tissue and membrane distribution and perform a variety of functions, such as the stabilization of trans-plasma membrane electrical potential, cell volume regulation and transepithelial transport [110]. Some of these CLCs have been linked to human inherited diseases, such as myotonia, kidney stones, and disorders associated with renal salt wasting and hypokalemic alkalosis ([114] and references therein).



	★	★★	★★★
CLC-0	GSGIPE	GKEGPFVH	PLAGVLF
CLC-5	GSGIPE	GKEGPLVH	PIGGVLF
CLC-6	GSGIPE	GKEGPMIH	PIGGTLF
Sc-CLC	GSGISE	GKEGPSVH	PIGGVLF
CLC-Nt1	GSGIPE	GKEGPMVH	PVGGVLF
CLC-Nt2	GPGIPE	GKEGPLVH	PVGGVLF
AtCLC-a	GPGIPE	GKEGPLVH	PVGGVLF
AtCLC-b	GPGIPE	GKEGPLVH	PVGGVLF
AtCLC-c	GSGIPE	GKEGPMVH	PVGGVLF
AtCLC-d	GSGIPE	GKEGPLVH	PVGGVLF
St-CLC	GSGIPE	GKEGPMVH	PVGGVLF
Ec-CLC	GSGIPE	GREGPTVQ	PLAGILF
Cyano-CLC	GGGIPQ	GRRAPTVH	PIAGVLF

Fig. 4. Topology model for the animal chloride channels (from [116]) and sequence alignment for three conserved domains (stars) in animal, yeast, plant and bacterial CLCs. CLC-0, *Torpedo marmorata*; CLC5 and CLC6, rat; ScCLC, *Saccharomyces cerevisiae*; CLC-Nt1 and CLC-Nt2, *Nicotiana tabacum*; AtCLC-a to -d, *Arabidopsis thaliana*; St-CLC, *Solanum tuberosum*; Ec-CLC, *Escherichia coli*; Cyano-CLC, *Synechocystis* sp.

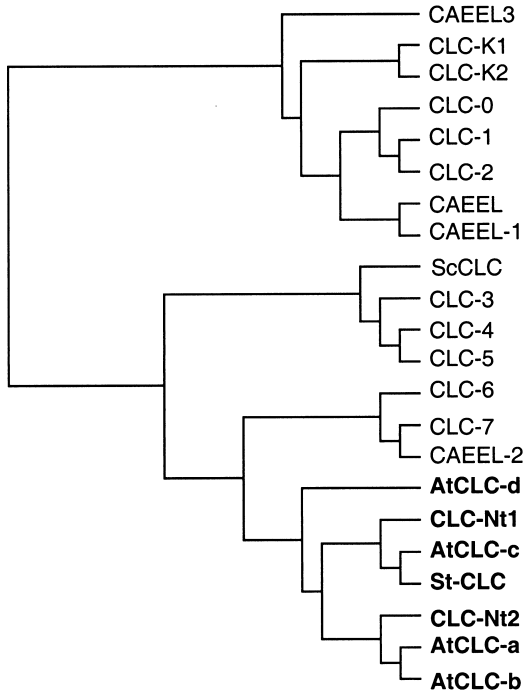


Fig. 5. Dendogram for some proteins of the CLC family: CLC-0 (P21564), from *Torpedo marmorata*; CLC-K1 (Q06393), CLC-K2 (D26111), CLC-1 (P35524), CLC-2 (P35525), CLC-3 (D17521), CLC-4 (X77197), CLC-5 (Z56277), CLC-6 (X83378), CLC-7 (Z67743), all from rat; CAEEL (U28943), CAEEL1 (U41551), CAEEL2 (Z75955), CAEEL3 (Z70037) from *Caenorhabditis elegans*; ScCLC (Z23117) from *Saccharomyces cerevisiae*; CLC-Nt1 (X95576) and CLC-Nt2 (Lurin et al., unpublished results) from *Nicotiana tabacum*; AtCLC-a (Z71445), AtCLC-b (Z71446), AtCLC-c (Z71447), AtCLC-d (Z71450) all from *Arabidopsis thaliana*; St-CLC (Y10338) from *Solanum tuberosum*. Plant CLCs are represented in boldface. The dendrogram was generated using pileup and growtree programs of GCG (Kimura protein distances, UPGMA).

To search for plant CLCs, Lurin et al. [108] used a PCR strategy based on amino acid motifs conserved in the four animal chloride channels known at this period, CLC-0 [111], CLC-1 [115], CLC-2 [116], CLC-K1 [117], and in the only CLC homolog from *Saccharomyces cerevisiae*, Gef1p or ScCLC [113]. This led to the cloning of two tobacco cDNAs, called *CLC-Nt1* [108] and *CLC-Nt2* (Lurin et al., unpublished results). Hechenberger et al. [109] also reported the cloning of four CLC cDNAs in *A. thaliana*, *AtCLC-a*, *AtCLC-b*, *AtCLC-c* and *AtCLC-d*, firstly identified as EST (expressed sequence tags) showing homology with animal and yeast CLC proteins.

CLC cDNAs cloned in tobacco or *Arabidopsis* code for proteins which share an identical size of about 800 amino acids (predicted MW of 85 kDa). Their hydropathy profiles exhibit specific hallmarks of the CLC family. Highly hydrophobic domains characteristic of intrinsic membrane proteins are similar in size and position to those of the animal channels and agree with the topological model proposed for animal CLC proteins (Fig. 4, [118]), with 10–12 transmembrane-spans and N-terminal and C-terminal domains localized in the cytoplasm [119]. Similar to their animal counterparts, plant CLCs show conserved amino acid sequence motifs (GxGxPE, GKxGPxxH, PxxGxLF) localized in the cytoplasmic loops between transmembrane domains D2–D3 and D5–D6 (Fig. 4) and thought for some of them to contribute to anion selectivity [120,121].

Sequence analysis revealed that the nine mammalian CLC proteins and the *Torpedo* fish CLC-0 can be divided into three subfamilies shown in the dendrogram of Fig. 5. The only CLC of the yeast *S. cerevisiae* (ScCLC, [113]) belongs to the same branch as CLC-3 [122], CLC-4 [123] and CLC-5 [124]. In contrast, the CLCs from bacteria (Ec-CLC, [112]) and cyanobacteria ([125], Cyano-CLC) define a separate fourth branch (not shown). It is noteworthy that all the plant CLC proteins belong to the same branch of the tree. They show about 35% identity with human CLC-6 and CLC-7 which belong to the same branch [126], but display only 25–30% identity with other CLC proteins.

4.2. Do plant CLCs encode functional anion channels?

Functional expression in heterologous systems has been widely used to study animal CLCs. For instance, the currents elicited in *Xenopus* oocyte after injection of CLC-0 and CLC-1 cRNAs show a strong similarity with those recorded in the corresponding native membrane of the protein, the plasma membrane of the electrical organ or the muscle cells, respectively [111,127]. *Xenopus* oocytes have been used for functionally characterizing plant CLCs. Expression of CLC-Nt1 [108] or CLC-Nt2 (Lurin and Maurel, unpublished results) led to hyperpolarization-activated inward currents corresponding to anion efflux across the oocyte membrane, which were modulated by extracellular anions and blocked

by external calcium and SITS. These results are consistent with the putative voltage-dependent chloride channel activity of the tobacco CLCs. However, the electrical currents elicited by these proteins in oocytes do not correspond to any of the currents described so far in plant cells, especially in the plasma membrane or the tonoplast. Moreover, the elicited currents observed with CLC-Nt proteins share similar properties with the currents induced by expression of different proteins (phospholemman, IsK protein) which were shown to activate oocyte endogenous channels [128]. Thus, at present, definite evidence for the genuine activity of tobacco CLCs remains to be provided.

None of the *Arabidopsis* cRNA of AtCLC-a, AtCLC-b, AtCLC-c or AtCLC-d elicited currents when injected in *Xenopus* oocytes, although it was demonstrated that corresponding AtCLC-GFP fusion proteins can be expressed in these cells [109]. Since animal CLC channels are known to function as homo- or hetero-multimers [129–131], Hechenberger et al. [109] tried to express different AtCLC proteins in combination, but with no more success than with each protein alone. Unknown subunits or regulatory proteins may also be lacking after heterologous expression. In order to identify interacting proteins, Weigmann et al. (unpublished results cited in [4]) used the N- and C-terminal cytoplasmic domains of the potato St-CLC as baits in the yeast two-hybrid system to screen a potato cDNA library, but no interacting protein could be detected. Other expression systems, such as Sf9 insect cells or Chinese hamster ovary (CHO) cells have been successfully used for animal CLCs [132–134] and could be envisaged for plant CLCs. To our knowledge, only one attempt has been reported by Weigmann et al. (cited in [4]) who mention as unpublished results their failure to express the potato St-CLC in insect cells.

Functional characterization based on the phenotypic complementation of a yeast *gef1* null-mutant [113] has been performed with *Arabidopsis* CLCs. GEF1 encodes the single CLC in yeast (ScCLC) and disruption of its gene leads to a defect in respiration and iron metabolism. Of all AtCLCs, only AtCLC-d [109,134] and AtCLC-c [134] were able to complement the *gef1* phenotype. This result would be compatible with an anion channel function for plant proteins, but several points remain to be clarified. To date, the anion channel activity of ScCLC is not fully demonstrated. In addition, the phenotype of *gef1* mutant is fully restored by the expression of AtCLC-d in haploid *gef1* cells [109] and by AtCLC-c and AtCLC-d in diploid cells [134], but not by AtCLC-a. Finally, CLC-0 is able to suppress all phenotypic traits of the *gef1* diploid mutant cells [134], whereas its function in the *Torpedo* electric organ is clearly different from that of GEF1 in yeast cells. Thus, the function of *Arabidopsis* CLC in the plant cell remains unclear and the cell localization of these proteins may provide hints at this.

Hechenberger et al. [109] showed that, when expressing fusion proteins of AtCLCs with green fluorescent protein (GFP) in yeast, all these proteins have an intracellular localization different from the vacuole membrane. In accordance with the phenotypic complementation, AtCLC-d colocalized with ScCLC-GFP, and the latter has been recently localized in the membrane of Golgi [135] and post-Golgi [134] vesicles. The critical point, however, remains to determine the intracellular localization of plant CLCs in their native cells. This question has been addressed by Lurin et al. (unpublished results) for tobacco ClC-Nt1 and CLC-Nt2 proteins. The use, in Western blot and immunocytochemistry experiments, of specific polyclonal antibodies raised against both proteins, showed that these proteins are preferentially expressed in an intracellular compartment, provisionally identified as the internal membrane of mitochondria. It thus appears that some of the plant CLC proteins have intracellular localizations, but this does not exclude the possibility that other members of the family reside in the plasma membrane or the tonoplast and can account for the channel activities described in these membranes.

4.3. Other gene families may encode plant anion channels

Anion channels different from those of the CLC family could also be responsible for the anion currents observed in the various plant cell types examined so far. For instance, Marten et al. [52] reported the existence in the guard cell plasma membrane of proteins immunologically related to p64, a component of chloride channels of plasma membrane and intracellular organelles in animal cells [136,137].

The superfamily of ATP binding cassette-containing (ABC) transporters regroups proteins that mediate the ATP-driven membrane translocation of a large variety of substrates [138]. A few members of this family exhibit an intrinsic anion channel activity. The most intensively studied is the cystic fibrosis transmembrane regulator (CFTR) which acts as a cAMP-regulated plasma membrane chloride channel [139,140], and when mutated, confers the autosomal recessive disorder cystic fibrosis. In the search for plant homologs of animal transport proteins, several genes encoding ABC transporters, but belonging to the subclass of the human multidrug resistance associated proteins (MRP) have been cloned in *Arabidopsis* ([141] and references therein; [142,143]). The role of these AtMRPs as active transporters for solutes involved in cell detoxification processes is well documented [144–146], except for one of them which is localized at the plasma membrane (AtPGP1, [147,148]). Until recently, no evidence was available concerning the existence of genuine plant CFTR homologs. A first indication was provided by pharmacological data demonstrating that CFTR-specific inhibitors were able to block the slow anion current from guard cells and to interfere with stomatal movements [149]. This led the authors to hypothesize that the molecular nature of the slow anion channel might be similar or related to that of the animal CFTR. The topic of plant ABC transporters will not be discussed in more detail in the present paper as it is covered by the review of Theodoulou in this volume [150].

5. From the functional and molecular characterization of anion channels to their physiological functions in the plant

5.1. A wide array of anion channel types achieves a great variety of physiological functions in plant cells

The few plant models described above illustrate the diversity of plant anion channels in terms of regulation mechanisms and physiological functions (Table 1). From these studies, it appears that a given cell type can be equipped with a variety of anion channels exhibiting distinct properties. These anion channels are of course expected to work in tight coordination with other ion transport proteins in the plasma membrane (ion channels, carriers and pumps), but also in intracellular membranes, such as the tonoplast.

A great deal of data, besides those discussed in this paper, also demonstrate that anion channels are present in numerous plant cell types and play a prominent role in many physiological responses. For instance, changes in the plasma membrane electrical potential and modulation of ion fluxes are among the most rapid alterations induced in plant cells by plant hormones (see for instance [151,152]), light [68,153]), osmotic variations [154], or signals issued from pathogenic as well as symbiotic microorganisms ([155–158] and references therein). In many cases, anion channel blockers were shown to interfere with these responses, not only by inhibiting early membrane depolarization and/or anion efflux, but further interfering with integrated growth [64,70] or defense responses [159,160]. Altogether, these data raise the idea that anion channels play a predominant role in the early steps of signaling pathways leading to developmental as well as adaptative plant responses. Besides this and a well-established role in cell turgor and volume regulation, plant anion channels participate in other functions for which their role needs to be elucidated in more detail. For instance, they contribute to the mineral nutrition of the plant, as a part of the integrated systems which allow the plant to take up nutrients from the soil and to translocate them from the root to the shoot. They likely play a role in cytosolic pH regulation and cell metabolism through their ability to make an electrical shunt for H^+-ATPases or to transport malate into the vacuole. Finally, another function for anion channels in intracellular membranes has been recently substantiated in animals and yeast. It was proposed, for instance, that the yeast CLC provides both the counterbalancing charge which allows cation compartmentalization into acidic vesicles [134,135], and the chloride ions required for the metal loading of proteins within this compartment [161]. Such functions are still totally unexplored in plant cells.

Whereas a lot of data has been accumulated concerning the plasma membrane, and to a lesser extent the vacuolar membrane, little is known yet on the

anion channel equipment of other internal membranes. Nevertheless, there has been some specific investigation on the membranes of plant mitochondria and chloroplast envelope. In both organelles, the outer membrane contains non-specific large pores whereas the inner membrane is the site of selective transport of metabolites and small ions. As in animals, the plant outer mitochondrial membrane contains porins, such as the VDAC (voltage-dependent anion-selective channel) showing a poor selectivity and very high conductance (reviewed in [162]). In contrast, anion-selective channels are present in the inner membrane of potato mitochondria [163] similar to what was found in heart and liver mitochondria [164]. Such channels may play a role in the regulation of membrane potential and/or mitochondrial volume. Anion channels in the chloroplast envelopes have also been characterized after reconstitution experiments [165], or directly in native membrane [166]. In the latter case, the permeation properties of the channel suggested that it could be responsible for the uptake of NO_2^- into the chloroplast and as such play an important role in nitrate assimilation. In chloroplasts, an anion-selective channel has been demonstrated in the photosynthetic thylakoid membrane [167,168]. This channel could mediate an uptake of anions in the thylakoids for maintaining the inner electroneutrality during massive light-driven H^+-uptake.

5.2. *Only a few genes coding for putative plant anion channels have been isolated*

To date, only a few plant genes, which all correspond to the same branch of the large CLC family have been isolated. Although the corresponding proteins display all structural features expected for CLCs, their function as anion channels has not been demonstrated unambiguously. Interestingly, the rapid-type anion channel from stomatal guard cells has pore properties and anion-dependent gating [36] which closely resemble those of the animal CLC-0 and CLC-1 [140]. As already discussed, further work is required to resolve the intracellular localization(s) and the function(s) of plant CLCs, and establish if any of the cloned genes may encode an anion channel already identified in electrophysiological studies. It has also been proposed that CFTR homologs are present in the plasma membrane of guard cells [149,169]. This idea, based on pharmacological evidence, also relies on the observation that the slow anion channel in these cells shares similar gating and regulation properties with the CFTR [45,140,169], but here again molecular data are needed to support this assumption.

Several strategies may be envisaged to search for new plant anion channel genes. It is expected that the sequencing of the *Arabidopsis* genome, which should be completed by the year 2001 [170], will provide a complete picture of gene families already identified, and will help to discover new gene families. More focused systematic approaches, such as the construction of cDNA libraries enriched in genes encoding plasma membrane or tonoplast proteins [171], or the use of a proteome strategy for tagging membrane proteins [172] can also constitute powerful resources to identify anion channel genes. Expression cloning, in yeast or *Xenopus* oocytes, would be another way to clone anion channels with a well-defined activity. Although *Xenopus* oocytes have been successfully used to isolate CLC-0, the first CLC member [111], this remains a difficult task mainly because of the various chloride channel activities present in the oocyte membrane, and because the level of mRNA encoding anion channels may be very low in plant tissues.

Finally, forward genetics remains an attractive strategy to identify novel channel genes. The screening for mutants altered in anion channel activity, with for instance a modified sensitivity to anion channel blockers, will have to be explored. On the other hand, it is expected that some of the mutants identified on the basis of a defect in cell signaling are mutated within anion channel genes, because of the fundamental role of anion channels in these processes.

5.3. *Reverse genetic approaches will permit the physiological role of anion channels to be explored in planta*

Once an anion channel gene has been identified, the next goal is to determine the physiological role of the corresponding proteins in plant tissues. Spatial and temporal patterns of protein or mRNA expression in the plant, at specific developmental stages or

in response to environmental stimuli can provide relevant clues to its biological function. In this respect, cell marking is a potent approach to correlate the expression of a gene in a specific cell type with the activity of the corresponding protein in planta. This approach was recently developed by Maathuis et al. [173] to study ion transport properties in specific cell types. These authors used transgenic plants expressing GFP under the control of the promoter of the ion channel gene to be studied, and fluorescent protoplasts were then assayed for channel activities using patch-clamp techniques.

A classical way is to transform plants with sense or antisense gene constructs and to study the phenotype resulting from the increase or decrease in the corresponding protein level. Recently, the use of knockout mutants in specific genes has opened new avenues to explore ion channel functions in the plant, as shown for *Arabidopsis* potassium channels [174,175]. Following a PCR-based strategy, our group screened an *Arabidopsis* T-DNA mutant library for the presence of insertions within AtCLC genes. One line was identified that carried a T-DNA insertion within the AtCLC-a gene (Geelen et al., unpublished results). Homozygous mutant plants did not exhibit any obvious morphological or developmental defect. However, measurements of anion concentrations revealed a 40–50% decrease specifically in the nitrate content of both root and shoot mutant tissues. In addition, mutant plants were hypersensitive to the herbicide chlorate, an analog of nitrate. The characterization of this AtCLC-a mutant thus opens new perspectives on the role of anion channels in the regulation of plant cell nitrate status. Further studies of this type, combining molecular, genetic and physiological approaches, will provide new insights into the general role of anion channels throughout plant development.

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