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REVIEW PAPER

The role of P-type IIA and P-type IIB Ca^{2+}-ATPases in plant development and growth

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Abstract

As sessile organisms, plants have evolved mechanisms to adapt to variable and rapidly fluctuating environmental conditions. Calcium (Ca^{2+}) in plant cells is a versatile intracellular second messenger that is essential for stimulating short- and long-term responses to environmental stresses through changes in its concentration in the cytosol ($[Ca^{2+}]_{cyt}$). Increases in $[Ca^{2+}]_{cyt}$ direct the strength and length of these stimuli. In order to terminate them, the cells must then remove the cytosolic Ca^{2+} against a concentration gradient, either taking it away from the cell or storing it in organelles such as the endoplasmic reticulum (ER) and/or vacuoles. Here, we review current knowledge about the biological roles of plant P-type Ca^{2+}-ATPases as potential actors in the regulation of this cytosolic Ca^{2+} efflux, with a focus the IIA ER-type Ca^{2+}-ATPases (ECAs) and the IIB autoinhibited Ca^{2+}-ATPases (ACAs). While ECAs are analogous proteins to animal sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPases (SERCAs), ACAs are equivalent to animal plasma membrane-type ATPases (PMCAs). We examine their expression patterns in cells exhibiting polar growth and consider their appearance during the evolution of the plant lineage. Full details of the functions and coordination of ECAs and ACAs during plant growth and development have not yet been elucidated. Our current understanding of the regulation of fluctuations in Ca^{2+} gradients in the cytoplasm and organelles during growth is in its infancy, but recent technological advances in Ca^{2+} imaging are expected to shed light on this subject.

Keywords: Autoinhibited Ca^{2+}-ATPase, *Arabidopsis thaliana*, ER-type Ca^{2+}-ATPase, P type, pollen tubes, root hairs.

Introduction

Plants use different ions to perform essential cellular processes, including metabolic activities that are critical for growth and development. Among them, calcium (Ca^{2+}) is an important nutrient and an essential cellular secondary signaling molecule. Plants have evolved efficient mechanisms to maintain and allow different gradients of cytosolic free Ca^{2+} and of Ca^{2+} stored in organelles such as the endoplasmic reticulum (ER), the Golgi apparatus, vacuoles, and plastids. For example, cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) are maintained in the sub-micromolar range, while in the vacuole and apoplast, Ca^{2+} concentrations are in the millimolar range (Stael *et al.*, 2012). Other studies have demonstrated that Ca^{2+} levels are in the sub-millimolar (50–500 μM) range in the ER (Stael *et al.*, 2012; Bonza *et al.*, 2013), 700 nM in the Golgi Apparatus (Ordenes *et al.*, 2012), 2 μM in the peroxisome (Drago *et al.*, 2008; Stael *et al.*, 2012), 100–600 nM in the mitochondrial matrix (Logan and Knight, 2003; Wagner *et al.*, 2015), and 80–150 nM in the chloroplasts and stroma (Loro *et al.*, 2016; Sello *et al.*, 2016). In response to different stimuli, specific and repetitive changes in $[Ca^{2+}]_{cyt}$, known as 'Ca^{2+} signatures' have been reported (Kudla *et al.*, 2010). The generation of these Ca^{2+} signatures as a response to different biotic and abiotic stresses, nutrient limitations, and developmental cues leads to activation of a number of diverse signaling pathways (Dodd *et al.*, 2010). Several groups of proteins can bind and respond to Ca^{2+}, such as Ca^{2+}-dependent protein kinases (CDPKs), calmodulins (CaMs), calmodulin-like proteins (CMLs), and calcineurin B-like proteins (CBLs). These can trigger downstream signaling responses that direct gene transcription, modify protein expression patterns, and induce metabolic changes that affect plant developmental and growth programs (Ranty *et al.*, 2016; Simeunovic *et al.*, 2016; Tang and Luan, 2017; Kudla *et al.*, 2018). In order to achieve Ca^{2+} homeostasis, plants must balance and maintain a transient high $[Ca^{2+}]_{cyt}$ during signaling events that is often followed by storage of Ca^{2+} in cellular compartments and/or release to the apoplast. In this review, we discuss the Ca^{2+} processes mediated by the P-type IIA ER-type Ca^{2+}-ATPases (ECAs) and the P-type IIB autoinhibited Ca^{2+}-ATPases (ACAs).

Plant Ca^{2+}-ATPases: ACAs and ECAs

In plant cells, three major classes of transporters control Ca^{2+} homeostasis, namely channels, exchangers, and pumps (i.e. ATPases). These Ca^{2+}-ATPases are involved in maintaining homeostasis by controlling Ca^{2+} efflux from the cytosol to organelles and/or to the apoplast. They are structurally similar in animals and plants. Plants contain P-type ATPases, which are additionally grouped as P-IIA ER-type Ca^{2+}-ATPases (ECAs) that are analogous to animal sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPases (SERCAs), and P-IIB autoinhibited Ca^{2+}-ATPases (ACAs) that are equivalent to animal PM-type ATPases (PMCAs) (Bonza and De Michelis, 2011). Fourteen P-type II Ca^{2+}-ATPases have been reported in the Arabidopsis genome, including four ECAs (AtECA1–4) and 10 ACAs (AtACA1, AtACA2, AtACA4, and AtACA7–13). Current knowledge of ECA and ACA functions in plant cells is fragmented and mostly comes from Arabidopsis. The most relevant information available on their subcellular localization, associated mutant phenotypes, and possible biological functions is summarized in Table 1.

Ca^{2+} efflux systems allow rapid removal of the excess $[Ca^{2+}]_{cyt}$ in order to end the signaling events, and also to prevent the harmful effects of having high $[Ca^{2+}]_{cyt}$ for long periods of time. Ca^{2+} exchangers (CAX) and Ca^{2+}-ATPases are the two main systems for removing Ca^{2+} from the cytoplasm. Ca^{2+}-ATPases are high-affinity (K_m=0.1–2 μM) but low-capacity transporters while CAXs are low-affinity (K_m=10–15 μM) but high-capacity transporters (Bose *et al.*, 2011). One of the main differences between ACAs and ECAs are their affinities for Ca^{2+}, which are in the micromolar range for ACAs and in the sub-micromolar range for ECAs (Bonza *et al.*, 2001; Meneghelli *et al.*, 2008). In addition, they have different specificities for divalent cations (Bonza and De Michelis, 2011): whereas ACAs are highly selective and transport only Ca^{2+}, ECAs can also transport Cd^{2+}, Mn^{2+}, and Zn^{2+} (Huda *et al.*, 2013; Kamrul Huda *et al.*, 2013). The molecular basis of these differences in specificity and affinity remains to be determined. Another important difference is that ECAs are specifically inhibited by cyclopiazonic acid (CPA) (Liang and Sze, 1998; Iwano *et al.*, 2009), whereas ACAs are particularly sensitive to inhibition by fluorescein derivatives such as erythrosin B or eosin Y (Eos) (Geisler *et al.*, 2000; Sze *et al.*, 2000; Bonza and De Michelis, 2011).

Most of the structural details of plant Ca^{2+} P-type ATPases have been inferred from their homologous animal SERCA proteins, for which several crystallized structures are available (Laursen *et al.*, 2009). At the protein level, both ACAs and ECAs contain three cytoplasmic domains, namely a P-domain (with the core Ca^{2+}-ATPase activity and phosphorylation), a nucleotide-binding domain (N-domain), and an actuator domain (A-domain), and two membrane domains, namely the transport (T-)domain and the class-specific support (S-). The ACAs, but not the ECAs, also contain an N-terminal autoinhibitory domain that binds calmodulin (CaM) and therefore activates the Ca^{2+} pump, thus allowing ACAs to be directly controlled by free Ca^{2+} levels. This N-terminal domain contains two sites to which the two Ca^{2+}-CaM molecules bind with different affinity; these sites are separated by eight amino acid residues so that there is no interaction between the two CaM molecules (Tidow *et al.*, 2012). The cytoplasmic A-, N-, and P-domains are essential in the hydrolysis of ATP, while the S- and T-domains transport ions (Palmgren and Nissen, 2011) (Supplementary Fig. S1 at *JXB* online). The P-domain is the catalytic center of Ca^{2+}-ATPase with the sequence DKTGTLT, in which the residue Asp (D) is the one that is phosphorylated during each active cycle (Fig. 1). The function of the N-domain, which is found within the P-domain, is to bind the ATP and to phosphorylate the P-domain. The structure of the N-domain is well conserved within the Ca^{2+}-ATPase family, although the length and sequence vary (KGAxE in ECA and KGAPE in ACA)

Table 1. Overview of Arabidopsis P-type IIA Ca^{2+}-ATPases (ECAs) and P-type IIB Ca^{2+}-ATPases (ACAs)

Gene name/ protein	Subcellular localization/ evidence	Functional evidence, mutant analysis, phenotypes	References
ECAs			
At1g07810/ ECA1	ER/ECA1 contains ER-retention motif, KxKxxECA1-GFP shows patterns similar to other ER markers	Complements yeast mutant (K616) defective in Ca^{2+} pumpsConfers tolerance to Mn2+ stressTransports Ca^{2+}, Mn^{2+}, and Zn^{2+}. Growth defect in *eca1* mutant under low Ca^{2+} and high Mn^{2+}	Chen *et al.* (1997); Liang *et al.* (1997); Hong *et al.* (1999); Wu *et al.* (2002); Dunkley *et al.* (2006); Li *et al.* (2008)
At4g00900/ ECA2	ER/ECA2 contains ER-retention motif, KxKxx	Transports Ca^{2+} and is involved in cellular metabolic process. Broad expression in roots	Baxter *et al.* (2003); Mills *et al.* (2008)
At1g10130/ ECA3	Golgi/ECA3 lacks ER-retention motif, KxKxx	*eca3-1* mutant shows reduced growth and chlorosis in the absence of Mn^{2+}. Involved in the homeostasis of Mn^{2+} and Ca^{2+}, and in transport of ions to Golgi apparatus	Mills *et al.* (2008); Li *et al.* (2008); Dunkley *et al.* (2006)
At1g07670/ ECA4	ER/ECA4 contains ER-retention motif, KxKxx	Responsible for Ca^{2+} ion transport, metabolic process. Broad expression in the roots	Baxter *et al.* (2003); Dunkley *et al.* (2006); Mills *et al.* (2008)
ACAs			
At1G27770/ ACA1	Chloroplast inner membrane/ immunodetection	Controls stomatal aperture, cytosolic distribution of chloroplasts in response to light, and root gravitropic curvature	Huang *et al.* (1993)
At4G37640/ ACA2	ER/sucrose gradients and expression of *p35s::ACA2-GFP*	Salt hypersensitivity in yeast, Ca^{2+}/calmodulin stimulated ATPase activity in yeast. Phosphorylation of Ca^{+2} pump ACA2 at Ser45 inhibits both basal and calmodulin-stimulated transport activities	Harper *et al.* (1998); Hong *et al.* (1999); Hwang *et al.* (2000) ; Anil *et al.* (2008)
At2G41560/ ACA4	Small vacuole/aqueous two-phase partitioning and expression of *p35s::ACA4-GFP*	ACA4 transcript is increased by NaCl, and when expressed in yeast it confers increased tolerance to NaCl. ACA4 adjusts cytosolic calcium concentrations by filling vacuolar compartments	Geisler *et al.* (2000); Sze *et al.* (2000); Boursiac *et al.* (2010); Gfeller *et al.* (2011)
At2G22950/ ACA7	Plasma membrane/ expression of *p35s::ACA7-GFP*	Involved in pollen development, particularly the progression from uni-nucleated microspores to bicellular pollen grains	Lucca and León (2012)
At5G57110/ ACA8	Plasma membrane/expression of *p35s::ACA8-GFP* and immunodetection	ACA8 mRNA level is up-regulated upon cold treatment. ABA increases the level of ACA8 protein at the plasma membrane. Role in sucrose signaling during early seedling development. Hypoxic treatments reduce the expression of ACA8. ACA8 is phosphorylated in response to flg22 and to the bacterial effector avrRpt2, and *in vitro* by CDPKs and by CIPKs	Bonza *et al.* (2000); Schiøtt and Palmgren (2005); Cerana *et al.* (2006); Benschop *et al.* (2007); Giacometti *et al.* (2012); Costa *et al.* (2017); Kadota *et al.* (2019)
At3G21180/ ACA9	Plasma membrane/expression of *pACA9::ACA9-GFP*	*aca9* displays reduced growth of pollen tubes and a high frequency of aborted fertilization. **ACA9** in young seedlings is stimulated by ABA	Sze *et al.* (2000); Axelsen and Palmgren (2001); Schiøtt *et al.* (2004); Cerana *et al.* 2006); Li *et al.* (2018)
At4G29900/ ACA10	Plasma membrane/expression of *p35s::ACA10-GFP*	mRNA level is down-regulated upon cold treatment. Involved in control of adult vegetative growth and Inflorescence structure. Plays a role in plant immunity	Sze *et al.* (2000); Axelsen and Palmgren (2001); Schiøtt and Palmgre (2005); George *et al.* (2008); Yang *et al.* (2017); Yu *et al.* (2018)
At3G57330/ ACA11	Vacuole/expression of *p35s::ACA11-GFP*	Involved in Ca^{2+} signaling and homeostasis. Acts as a genetic suppressor of the programmed cell death pathway in plants	Lee *et al.* (2007); Boursiac *et al.* (2010)
At3G63380/ ACA12	Plasma membrane	Unlike other ACAs, ATPase activity in yeast is not stimulated by calmodulin. High expression upon flagellin treatment	Frei dit Frey *et al.* (2012); Limonta *et al.* (2014); Yu *et al.* (2018)
At3g22910/ ACA13	Plasma membrane and vesicles/ expression of *pACA13::ACA13-Venus*	ACA13 is induced 34-fold in response to pathogen stressACA13 contributes to pollen germination. ACA13 is induced by osmotic stress. *aca10aca13* mutants exhibit a severe reduction in seed number	Boursiac and Harper (2007); Iwano *et al.* (2014); Yu *et al.* (2018)

(Fig. 1, Supplementary Fig. S1). The A-domain, the smallest of the cytoplasmic domains, places the highly conserved Thr-Gly-Glu (TGE) sequence over the phosphorylated P-domain, thus protecting the two high-energy phosphate bonds against spontaneous hydrolysis (Bublitz *et al.*, 2011) (Fig. 1). The T-domain has six transmembrane segments; it is a very flexible domain that moves during the catalytic cycle due to the association–dissociation cycle of the ions. It is structurally

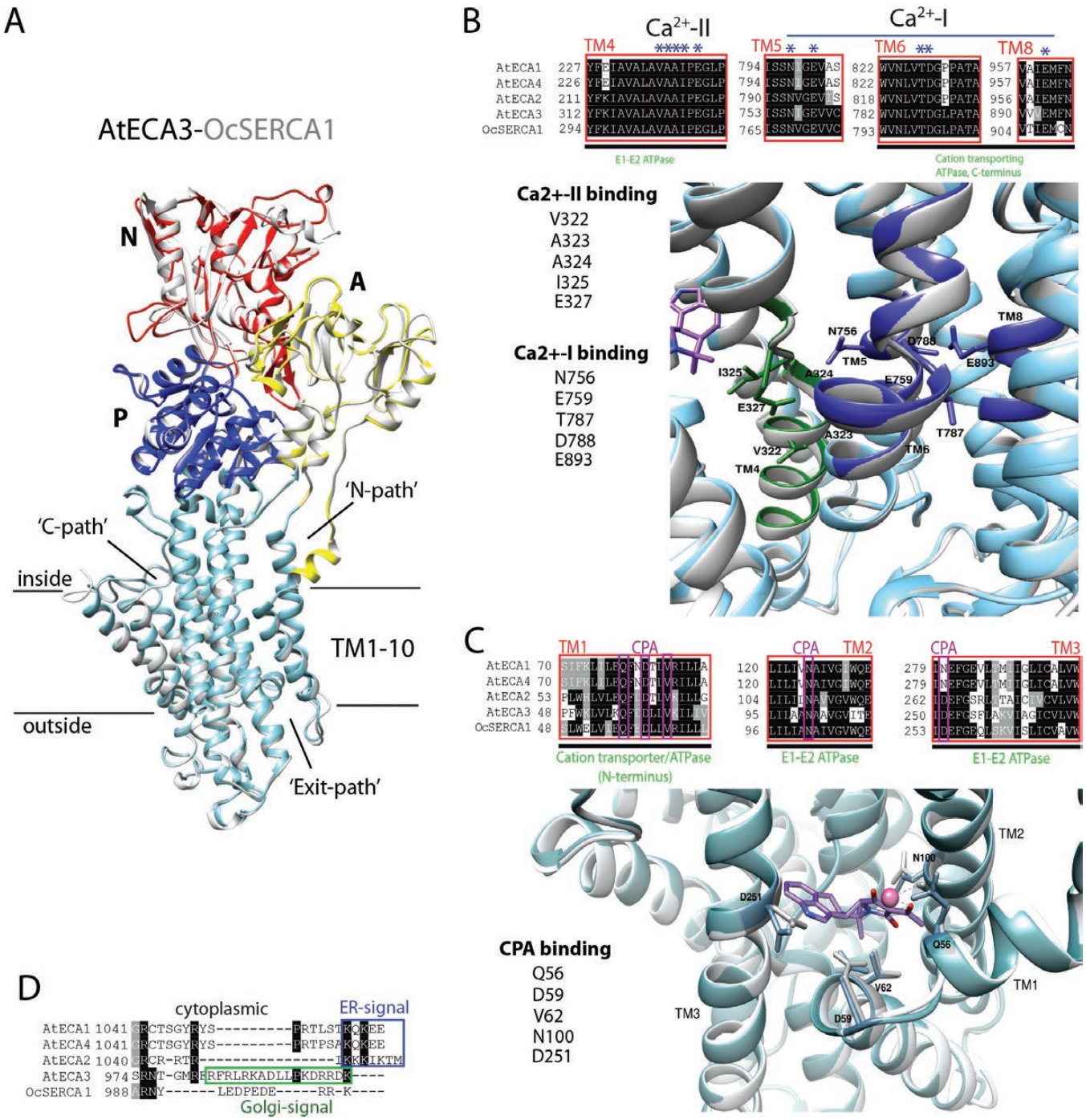

Fig. 1. Structure and conservation of Arabidopsis Ca^{2+}-ATPase type IIA ACA3 (AtECA3) according to the Protein Data Bank (https://www.rcsb.org/). (A) Ribbon diagram of the ACA3 model, showing a typical P-type A superimposed on its template (grey PDB code: 3FGO). The different domains are displayed in different colors. 'Inside' refers to the cytoplasm and 'outside' to intracellular compartments (ER/Golgi). The N-, C-, and Exit-paths are indicated by lines. (B) Sequence conservation of Ca^{2+}-binding residues of the AtECA3 model and *Oryctolagus cuniculus* OcSERCA1a, and illustration of the transmembrane region showing Ca^{2+}-I (green) and Ca^{2+}-II (blue) binding sites. Residues indicated in the alignment are labeled in the illustration and represented by bars. (C) Sequence conservation of cyclopiazonic acid (CPA)-binding residues among P-type ATPases OcSERCA1a and AtECAs, and illustration of CPA binding to the AtECA3 model. Residues highlighted in violet boxes in the alignment are mapped onto the structure (represented as bars). CPA is also represented by bars. The protein is rotated to give the best view of the binding sites. The figure was generated using Chimera (Pettersen *et al.*, 2004). (D) C-terminal sequences of OcSERCA1a and ECAs from Arabidopsis. ER-retention (KDEL) and Golgi signals are indicated.

supported by the S-domain, which also offers side-chains for additional ion-binding sites.

ACAs and ECAs are involved in plant development and adaptation to the environment through rapid changes in $[Ca^{2+}]_{cyt}$ in response to different stimuli (Huda *et al.*, 2013). These changes establish an equilibrium between the influx and efflux of the ion. Influx is regulated by a wide variety of different membrane channels such as ligand (cyclic nucleotide

and amino acid)-gated channels, stretch-activated channels, and voltage-dependent channels (Costa *et al.*, 2018). To down-regulate the duration and strength of the specific stimulus, cells need to lower the $[Ca^{2+}]_{cyt}$ by moving Ca^{2+} to the apoplast or by storing it in the ER or vacuole. Using a Cameleon variant Ca^{2+} sensor (CRT-D4ER) that monitors ER luminal Ca^{2+} *in vivo*, it has been shown that the accumulation of Ca^{2+} in the ER follows the increases in cytosolic Ca^{2+} that are triggered by the different stimuli (Bonza *et al.*, 2013). This suggests that the ER may function as a buffer against transient increases in $[Ca^{2+}]_{cyt}$. However, more work is required to understand whether global Ca^{2+} signatures are coordinated between the cytoplasm and ER. With respect to the Golgi apparatus, it is technically challenging to measure Ca^{2+} levels dynamically, since this a highly mobile organelle. Through the use of the bioluminescent Ca^{2+} reporter aequorin, it has been shown that storage in the Golgi does not contribute to the pool of $[Ca^{2+}]_{cyt}$, although its Ca^{2+} homeostasis is necessary for post-translational protein modification and secretion (Ordenes *et al.*, 2012).

ECAs maintain Ca^{2+} and Mn^{2+} homeostasis in the ER and Golgi compartments

In contrast to ACAs, little is known about the functions and regulation of ECAs. ECA1, ECA2, and ECA4 are predicted to localize in the ER, since they contain an ER-retention motif (KxKxx) in their C-terminal sequences (Dodd *et al.*, 2010) (Table 1, Fig. 1D, Supplementary Fig S1). This has been confirmed by proteomic studies based on organelle-enriched fractions (Dunkley *et al.*, 2006) and by co-localization studies using transient expression of ECAs coupled with confocal microscopy and ER fluorescent markers (Liang *et al.*, 1997). By contrast, ECA3 contains a C-terminal Golgi signal that is rich in basic amino acid residues (KDRRDK), and similar co-localization studies have shown it to be targeted to the Golgi apparatus and early trans-Golgi network endosomes (Baxter, 2003; Mills *et al.*, 2008). ECA3 might be important in the regulation of Ca^{2+} levels in the Golgi apparatus, which is highly sensitive to treatment with CPA (Ordenes *et al.*, 2012). Further studies with an *eca3* null-mutant would be needed to confirm this hypothesis. To date, no ECAs have not been found in the plasma membrane or in any other organelle; however, other subcellular locations cannot be excluded (Ferrol and Bennett, 1996; Downie *et al.*, 1998).

Calcium gradients are relevant in polar-growing cells such as pollen tubes and root hairs (Konrad *et al.*, 2011). Waese *et al.* (2017) examined expression patterns of ECA1–4 in root epidermal cells, during pollen development and in pollen-tubes using the ePlant database and a tissue-specific eFP browser (http://bar.utoronto.ca/eplant). Most of the ECAs (except ECA2) are highly expressed in the early stages of root epidermis cell development while ECA3 and ECA4 are present at high levels only during root elongation (Fig. 2A). This suggests that ECAs only have a significant role in regulating the Ca^{2+} gradient during differentiation of atrichoblasts and trichoblasts and at very early stages of root hair development. In contrast,

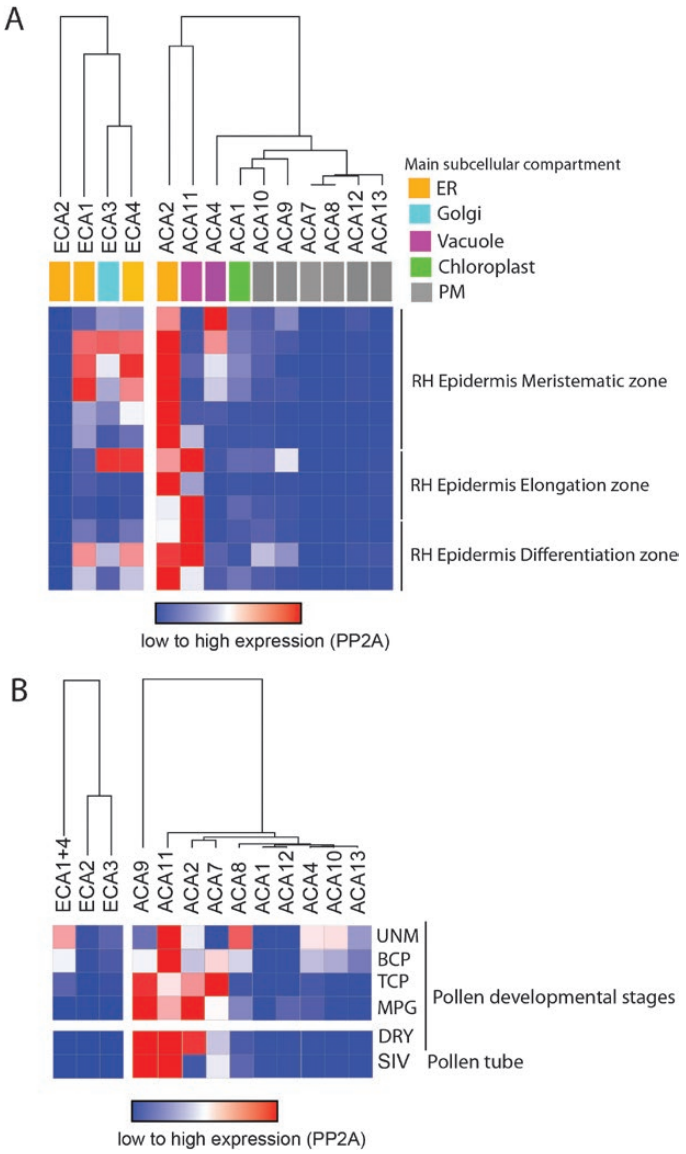

Fig. 2. Expression patterns of Arabidopsis P-type IIA Ca^{2+}-ATPases (ECAs) and P-type IIB Ca^{2+}-ATPases (ACAs) in (A) root epidermal cells and (B) in pollen. Expression values are normalized to the housekeeping gene *PP2A* and values are shown in as heat-maps. Subcellular localizations are detailed in Table 1. PM, plasma membrane; RH, root hair; UNM, uninuclear microspores; BCP, bicellular pollen; TCP, tricellular pollen; MPG, mature pollen grains; DRY, dry pollen grains; SIV, semi *in vivo* pollen germination.

very low or almost no expression of ECAs is detected during pollen development and in pollen tubes (Fig. 2B).

Several ECAs transport not only Ca^{2+} but also other cations such as Mn^{2+} and Zn^{2+}. It has been shown that under elevated Mn^{2+}, ECA1 and ECA3 restore growth defects of the yeast *pmr1* mutant, which is defective in a Golgi Ca^{2+}/Mn^{2+} pump; this confirms their role in the homeostasis of Mn^{2+} and Ca^{2+}, and in the transport of these ions to endomembrane compartments (ECA1; Wu *et al.*, 2002) and to the Golgi (ECA3; Mills *et al.*, 2008). Overall, the involvement of ECAs in Ca^{2+} homeostasis has been found to be stress responsive. Thus, the expression of *Triticum aestivum* (wheat) *TaECA2A* and *TaECA2B* is up-regulated after heat and drought stress

treatments (Taneja and Upadhyay, 2018), while the ortholog in *Oryza sativa* (rice), *OsECA1*, shows elevated expression upon exposure to drought conditions (Kamrul Huda *et al.*, 2013) and also as a result of induction by gibberellin in the aleurone layer (Chen *et al.*, 1997). All current evidence suggests that ECAs play a major role in ER–Golgi-linked Ca^{2+} homeostasis related to abiotic stress responses. It has recently been shown that Mizu-Kussey1 (MIZ1), a protein of unknown function associated with the ER membrane, interacts with and inhibits ECA1 to balance the cytosolic Ca^{2+} influx and efflux required for root bending towards water (Shkolnik *et al.*, 2018). This implies that a tight regulation of ECA activities might exist in plant cells during diverse developmental and physiological processes. Further studies are needed to establish the molecular mechanisms that control each of the four ECAs in different biological contexts.

The transport mechanism of plant ECAs has not yet been determined. The animal type-IIA Ca^{2+}-ATPase SERCA is known to transport two Ca^{2+} ions per catalytic cycle, in exchange with two H^+ ions (Obara *et al.*, 2005; Brini *et al.*, 2013). The two Ca^{2+} ions are coordinated by six amino acids located in the transmembrane domains (TMs) TM4, TM5, TM6, and TM8, three of which are also involved in H^+ translocation (Obara *et al.*, 2005; Møller *et al.*, 2010; Brini *et al.*, 2013). Arabidopsis ECAs have good overall similarity in their protein sequences with animal SERCAs (~50–54% identity), and all residues responsible for Ca^{2+} binding in rabbit (*Oryctolagus cuniculus*) OcSERCA1a are fully conserved in Arabidopsis ECAs with the same orientation (Fig. 1B, Supplementary Fig. S1). Based on this, it is likely that Arabidopsis ECAs also transport two Ca^{2+} ions per catalytic cycle, in exchange with protons. Given that all Arabidopsis ECAs share most of the amino acids involved in the binding to CPA with animal SERCAs (Fig. 1C, Supplementary Fig. S1), it can be postulated that they may be inhibited by CPA as well. It is proposed that CPA inhibits SERCAs by blocking the Ca^{2+} access channel and immobilizing a subset of transmembrane helices in a non-native conformation that is incompatible with Ca^{2+} binding and transport (Moncoq *et al.*, 2007). Indeed, CPA inhibition of ECAs has been experimentally validated (Iwano *et al.*, 2009). However, unlike SERCAs, ECAs are insensitive to the non-competitive thapsigargin, possibly because ECAs lack the conserved binding site located between the TM3–TM8 domains (Liang and Sze, 1998; Obara *et al.*, 2005; Brini *et al.*, 2013). Further studies are required to validate the molecular mechanism of Ca^{2+} transport by ECAs in plant cells.

ACAs are involved in plant development, stress responses, and pollination

Although ACAs can be grouped into four clusters based on their amino acid sequences (Yu *et al.*, 2018), their expression patterns and roles in plant growth are more complex (e.g. Table 1). Four ACAs (ACA2, 7, 9, 11) are highly expressed during most of the pollen developmental stages (Fig. 2), ACA7, 9, and 11 are expressed in pollen tubes (which is in contrast to ECAs), and ACA2 and ACA11 are also found in root epidermal cells, including growing root hairs. This clearly indicates an important role of ACAs in polar-growing cells. The expression patterns of ACAs correlate with the phenotypes in some of the mutants that have been characterized. Multiple mutations in *ACA8*, *ACA10*, and *ACA13* lead to severe, but different, phenotypes during vegetative growth (Table 1). ACA7 and ACA9 are found in pollen (Schiøtt *et al.*, 2004; Lucca and León, 2012), while ACA13 is expressed in the papillary cells of the stigma (Iwano *et al.*, 2014). Insertional mutants of *ACA9* show reduced pollen tube growth and defects in fertilization that result in a semi-sterile phenotype (Schiøtt *et al.*, 2004); however, the exact role of ACA9 during pollen tube growth is unknown. ACA13 may be involved in providing Ca^{2+} to compatible pollen tubes and thereby initiating proper growth through the pistil (Iwano *et al.*, 2014).

Different stress stimuli are known to trigger rapid changes in $[Ca^{2+}]_{cyt}$ (Huda *et al.*, 2013) and it is therefore not surprising that ACAs are involved in various stress responses. For instance, ACA2 and ACA4 are able to alleviate hypersensitivity to salt in yeast by controlling $[Ca^{2+}]_{cyt}$ (Anil *et al.*, 2008). ACA4 and ACA11 are involved in the defence response; the *aca4 aca11* double-mutant shows enhancement of the hypersensitive response through the activation of the salicylic acid signaling pathway (Boursiac *et al.*, 2010). ACA8 and ACA10 interact with the receptor kinase FLAGELLIN SENSITIVE2 (FLS2), and *aca8 aca10* double-mutants show decreases in cytosolic Ca^{2+} and in bursts of reactive oxygen species in response to the bacterial flagellin flg22 (Frei dit Frey *et al.*, 2012). ACA8 is phosphorylated in response to flg22 (Benschop *et al.*, 2007) and to the bacterial effector avrRpt2 (Kadota *et al.*, 2019), and *in vitro* is phosphorylated by CPK1 and CPK16, two calcium-dependent protein kinases (Giacometti *et al.*, 2012), and by CIPK9 and CIPK14, two CBL-interacting protein kinases (Costa *et al.*, 2017), all of which suggest a role of ACA8 in plant immunity. ACA10 also plays an important role in defence responses, together with ACA8, ACA12, and ACA13 (Yu *et al.*, 2018). The combined role of ACA8 and ACA10 in immune signaling was confirmed by the observation that the evolutionarily conserved protein BON1 interacts with ACA10 and ACA8 to control stomatal movement and plant immunity (Yang *et al.*, 2017). It has been reported that paralogs of BON1 (BON2 and BON3) interact with ACA10 and ACA8, and also with the pollen-specific ACA9, and the triple-mutant *bon1 bon2 bon3* shows defects in pollen germination and seed production (Li *et al.*, 2018).

The different subcellular localizations of ACAs may provide plant cells with several ways of controlling diverse Ca^{2+} signals, thereby specifying alternative mechanisms for triggering precise Ca^{2+} signatures. Several studies of the subcellular location of ACA proteins have been performed in heterologous systems using strong promoters such as 35S and these results should therefore be interpreted carefully. While ACA1 is localized to the inner envelope of chloroplasts (Huang *et al.*, 1993), ACA2 is localized in the ER (Hong *et al.*, 1999) and ACA4 is found in the membrane of small vacuoles (Geisler *et al.*, 2000). ACA 11 is localized in the large central vacuole (Lee *et al.*, 2007). The remaining ACAs (ACA7–10, ACA12, ACA13) are localized to the plasma membrane (Bonza *et al.*, 2000; Lucca and León, 2012; Limonta *et al.*, 2014; Costa *et al.*, 2017; Yang *et al.*, 2017)

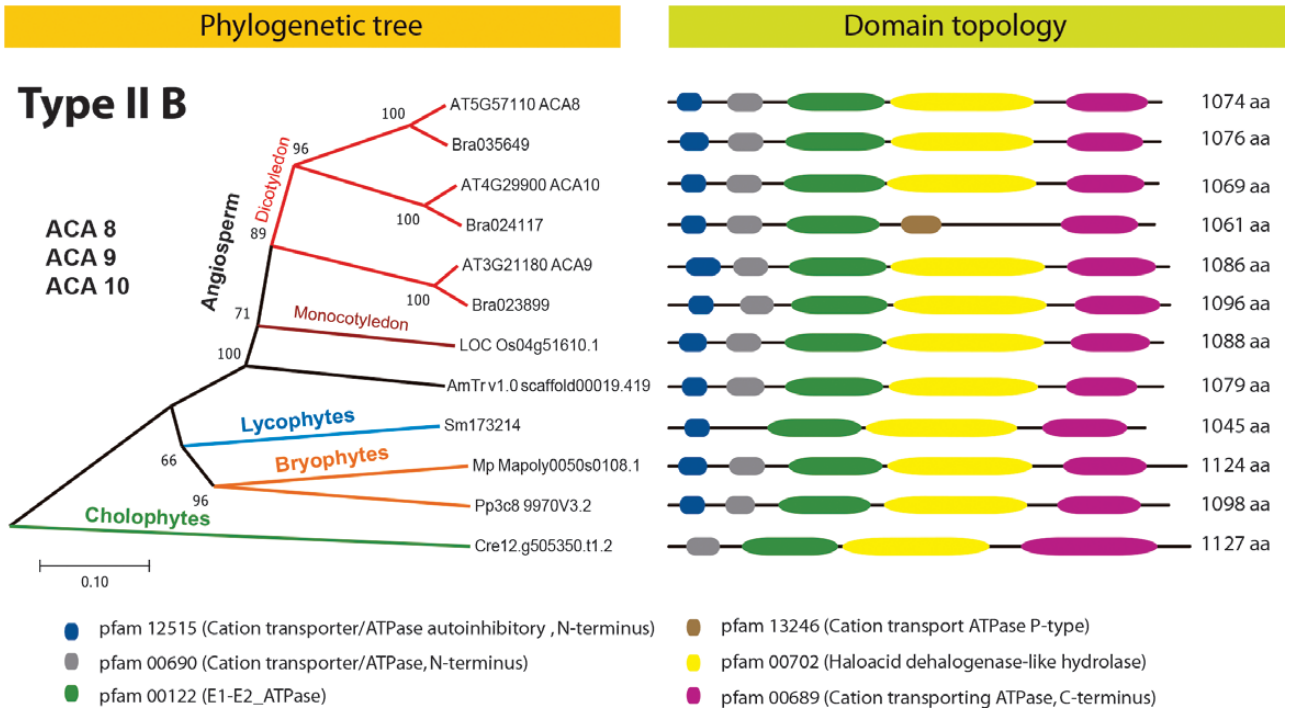

Fig. 3. Molecular phylogenetic analysis and representation of domain structures of the ACA8–ACA9–ACA10 clade of ACAs. The numbers next to the branches show the significant percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). For each amino acid sequence a schematic domain is shown based on the results of Pfam. AmT, *Amborella trichopoda*; AT, *Arabidopsis thaliana*; Bra, *Brassica napus*; Cr, *Chlamydomonas reinhardtii*; Mp, *Marchantia polymorpha*; Os, *Oryza sativa*; Pp, *Physcomitrella patents*; Sm, *Selaginella moellendorffii*.

.The localization patterns of ACAs and ECAs are summarized in Table 1 and in Fig. 2.

The following ACAs all function as calcium pumps in the mutant yeast strain K616, in which endogenous calcium ATPases are disrupted: ACA2 (Harper *et al.*, 1998), ACA4 (Geisler *et al.*, 2000), ACA8 (Bækgaard *et al.*, 2006; Giacometti *et al.*, 2012), ACA9 (Schiøtt *et al.*, 2004), ACA11 (Lee *et al.*, 2007), ACA12 (Limonta *et al.*, 2014), and ACA13 (Iwano *et al.*, 2014). More studies are needed to verify that Ca^{2+} transport is indeed carried out by ACAs and ECAs in plants.

Evolution of ACAs and ECAs

In an attempt to understand the evolution of plant P-type II Ca^{2+}-ATPases, we have examined the conservation of their amino acid sequences in the following plant species: *Chlamydomonas reinhardtii* (Chlorophytae), *Marchantia polymorpha* (bryophyte), *Physcomitrella patens* (bryophyte), *Selaginella moellendorffii* (lycophyte), *Amborella trichopoda* (angiosperm), *O. sativa* (monocot), *Brassica napus* (dicot), and *Arabidopsis thaliana* (dicot). For each of the 14 Arabidopsis ECA and ACA protein sequences, we searched for homologs in each of the listed species and selected the sequence with the closest similarity. For some species the same homologous sequence was obtained for different Arabidopsis P-type Ca^{2+}-ATPases, with the result that a total of only 58 full-length sequences were obtained from the Phytozome (https://phytozome.jgi.doe.gov/) and NCBI (https://www.ncbi.nlm.nih.gov/) databases after eliminating duplicates. The ACA and ECA sequences formed two clearly distinct groups (Supplementary Fig. S2), indicating that they have evolved separately. Our phylogenetic comparison of the protein sequences indicated that the closer the species was to Arabidopsis, the higher the level of identity with its homolog, which suggests a functional conservation. Not surprisingly, in the less-evolved species (*C. reinhardtii*, *M. polymorpha*, *P. patens*, and *S. moellendorffii*) one sequence in each of them matched more than one sequence of the Arabidopsis P-type IIA or IIB Ca^{2+}-ATPases (Fig. 3, Supplementary Fig. S2), suggesting that in these species, the function of the Ca^{2+}-ATPases could be assigned to other proteins that were not similar to Arabidopsis ACAs and ECAs. For example, for all four Arabidopsis ACA groups only two putative homologs were found for *M. polymorpha* and *S. moellendorffii*, and three for *P. patens*. Moreover, only three *C. reinhardtii* sequences (Cre12.g505350.t1.2, Cre02.g145100.t1.1, Cre16.g681750.t1.1), which were grouped together, matched the 10 Arabidopsis ACAs with a sequence identity of 40–46% (Supplementary Fig. S2). For the four Arabidopsis ECAs there were two *C. reinhardtii* sequences, one for each ECA sub-group with a sequence identity of 59–60%. Taking these results together, we can conclude that there is greater variation among *C. reinhardtii* homologs for ECAs than for ACAs, which suggests that ECAs may have appeared before ACAs during the evolution of the green lineage. A recent study of the evolution of the P-type ATPase (P-ATPase) Superfamily that was performed on a wider range of eukaryotic groups (from Chloroplastida to Haptophyta) showed that the sarco/

endoplasmic reticulum Ca^{2+} ATPase (SERCA, P2A) and the P5A ATPase genes were duplicated very early in eukaryotic evolution and before the divergence of the present eukaryotic supergroups such as the SAR clade (Stramenopiles, Alveolata, Rhizaria, Cryptophyta, and Haptophyta) (Palmgren *et al.*, 2020). The sequences of the proteins of the two main P2A clades (P2A-I where AtECA3 is located, and P2A-II where AtECA1, AtECA2, and AtECA4 are located) diverge at some specific points in the P-domain and in the TM5 and TM6 domains (Palmgren *et al.*, 2020). Although these changes are observed at the amino acid level, the possible functional and/or structural implications have not yet been tested experimentally.

In order to examine the divergence of the ACAs in detail, we specifically studied the phylogenetic divergence of the subgroup ACA8–10 (Fig. 3). As expected, our phylogenetic analysis based on the ACA protein sequences followed the evolution of land plants (Bowman *et al.*, 2017). Only one homologous sequence of *M. polymorpha* (Mapoly0050s0108.1), *P. patens* (Pp3c8 9970V3.2), and *S. moellendorffii* (Sm173214) matched ACA8, ACA9, and ACA10. It is also worth noting that while the rice genome is three times larger than that of Arabidopsis, rice had only one sequence (LOC_Os04g516110) with high identity with AtACA8 (72%), AtACA9 (70%), and AtACA10 (70%), suggesting a common ancestry of these proteins in these two species. However, due to their duplication history, it can be also inferred that rice has other types II Ca^{2+}-ATPases with less identity to Arabidopsis (Treesubsuntorn and Thiravetyan, 2019).

The main difference between ACAs and ECAs is the presence of an N-terminal calmodulin-binding autoinhibitory domain only in the ACAs (Huda *et al.*, 2013). Among all the species analysed here, the N-terminal ACA domain (Pfam 12 515) was conserved except in *C. reinhardtii* (Fig. 3). This observation is consistent with the idea that the C-terminus domain of ACAs is related to less-evolved species. To examine the conservation of all the protein domains of the clade of type IIB (ACA8–10), we analysed the alignment of the partial sequences of each domain using the Pfam software (Finn *et al.*, 2016). The LOGO sequence view of the consensus pfam domains of ACA8-10 suggested a strong conservation of each motif for all species (Supplementary Fig. S3).

Future research and challenges

Over the past two decades, significant, albeit somewhat fragmented, progress has been made in understanding the Ca^{2+} transport in multiple subcellular compartments that is mediated by ACAs, and to a lesser extent by ECAs. A complex picture is starting to emerge of a multifactorial network that regulates the cytoplasmic and organellar Ca^{2+} signatures. Further studies of ACA and ECA members are now required to advance our understanding of the global regulation system. Major breakthroughs in our understanding of how Ca^{2+} signatures are regulated in plant cells can be anticipated in the near future, due to developments such as genetically encoded fluorescence Ca^{2+} reporters that are targeted to different subcellular compartments (Krebs *et al.*, 2012; Loro *et al.*, 2012, 2016) and improved fluorescence microscopy techniques, including light sheet fluorescence microscopy (LSFM) and selective plane illumination microscopy (SPIM) (Maizel *et al.*, 2011; Costa *et al.*, 2013; Candeo *et al.*, 2017). Based on these technical improvements, it may soon be possible to use fluorescent compatible multi-organellar sensors to simultaneously track Ca^{2+} dynamics in the cytoplasm/ER, in the cytoplasm/Golgi apparatus, and in the cytoplasm/vacuole compartments in response to stress stimuli in wild-type plants as well as in single and multiple mutants of *aca* and *eca*. This should provide a more complete picture of how ECAs and ACAs contribute to the formation of intracellular reservoirs for the pool of $[Ca^{2+}]_{cyt}$.

Supplementary data

Supplementary data are available at *JXB* online

Fig. S1. Protein domains and alignment of Arabidopsis AtECA1–AtECA4.

Fig. S2. Molecular phylogenetic analysis of plant ACAs and ECAs by the maximum likelihood method.

Fig. S3. LOGO view of the consensus pfam domains for the clade ACA8–10 for all the species studied.

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Author contributions

JGB, KK, and MLB reviewed the text, references, and figures; GDD and YCRG reviewed the text; CMB performed the molecular modelling of ECA3; MO, JPM, and JME conceived the project, designed the figures, and wrote the article with contributions from all the authors.

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