Chloroplast Calcium Signaling in the Spotlight

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Calcium has long been known to regulate the metabolism of chloroplasts, concerning both light and carbon reactions of photosynthesis, as well as additional non photosynthesis-related processes. In addition to undergo Ca²⁺ regulation, chloroplasts can also influence the overall Ca²⁺ signaling pathways of the plant cell. Compelling evidence indicate that chloroplasts can generate specific stromal Ca²⁺ signals and contribute to the fine tuning of cytoplasmic Ca²⁺ signaling in response to different environmental stimuli. The recent set up of a toolkit of genetically encoded Ca²⁺ indicators, targeted to different chloroplast subcompartments (envelope, stroma, thylakoids) has helped to unravel the participation of chloroplasts in intracellular Ca²⁺ handling in resting conditions and during signal transduction. Intra-chloroplast Ca²⁺ signals have been demonstrated to occur in response to specific environmental stimuli, suggesting a role for these plant-unique organelles in transducing Ca²⁺-mediated stress signals. In this mini-review we present current knowledge of stimulus-specific intra-chloroplast Ca²⁺ transients, as well as recent advances in the identification and characterization of Ca²⁺-permeable channels/transporters localized at chloroplast membranes. In particular, the potential role played by cMCU, a chloroplast-localized member of the mitochondrial calcium uniporter (MCU) family, as component of plant environmental sensing is discussed in detail, taking into account some specific structural features of cMCU. In summary, the recent molecular identification of some players of chloroplast Ca²⁺ signaling has opened new avenues in this rapidly developing field and will hopefully allow a deeper understanding of the role of chloroplasts in shaping physiological responses in plants.

Keywords: chloroplasts, organelar calcium signaling, calcium-permeable channels, calcium transporters, calcium binding proteins, genetically encoded calcium indicators, chloroplast calcium uniporter

INTRODUCTION

Calcium is a fundamental intracellular messenger involved in a wide range of different signaling pathways in all eukaryotes. In plants, Ca²⁺ has been shown to participate in the transduction of a large variety of environmental stimuli of both abiotic and biotic nature (Dodd et al., 2010). A complex Ca²⁺ homeostatic and signaling machinery allows for a tight regulation of the intracellular concentration of the ion ([Ca²⁺]) and its variations during signal transduction (Kudlà et al., 2018). Plant organelar Ca²⁺ signaling is a rapidly expanding field of investigation, also thanks to the
increasing availability of novel genetically encoded Ca\textsuperscript{2+} indicators, specifically targeted to different intracellular compartments (Costa et al., 2018). In addition to the vacuole, considered as the main stimulus-releasable Ca\textsuperscript{2+} store in the plant cell, other organelles, i.e. chloroplasts, have recently come to the fore. The detection of stimulus-specific intra-chloroplast Ca\textsuperscript{2+} signals in response to different environmental cues has highlighted the contribution of chloroplasts to shaping cytosolic Ca\textsuperscript{2+} signatures. In this mini-review we present the most recent research works dealing with the monitoring of chloroplast Ca\textsuperscript{2+} concentration and its changes during signal transduction events. Moreover, we focus on the recently reported identification and biochemical characterization of some molecular players involved in chloroplast Ca\textsuperscript{2+} handling. Current evidence for a crucial role of chloroplasts as stress sensors and future avenues of investigation in this promising field are also discussed.

**THE EMERGING ROLE OF CHLOROPLAST CALCIUM SIGNALING IN THE TRANSDUCTION OF BIOTIC AND ABIOTIC STRESS SIGNALS**

Chloroplasts have long been known to be involved in intracellular Ca\textsuperscript{2+} homeostasis and signaling. The regulatory role played by these organelles on intracellular Ca\textsuperscript{2+} handling is two-fold: i) a tight control of intra-organellar [Ca\textsuperscript{2+}] is essential for the proper functioning of the chloroplast physiology, e.g. the regulation of photosynthesis, as well as other chloroplast-localized processes (Stael et al., 2012b; Rocha and Vothknecht, 2012; Nomura and Shiina, 2014; Hochmal et al., 2015); ii) transient changes in stromal [Ca\textsuperscript{2+}] ([Ca\textsuperscript{2+}]\textsubscript{str}), evoked in response to different stress stimuli, in turn can shape intracellular Ca\textsuperscript{2+} signals, thereby affecting Ca\textsuperscript{2+}-mediated signaling circuits.

After the pioneering work conducted by Johnson et al. (Johnson et al., 1995) and Sai and Johnson (Sai and Johnson, 2002), who monitored [Ca\textsuperscript{2+}] in the chloroplast stroma by means of a chloroplast-targeted aequorin chimera, precise measurements of Ca\textsuperscript{2+} levels inside the different chloroplast subcompartments have been lacking for a long time. However, in the last few years the increasing availability of specifically targeted Ca\textsuperscript{2+} reporters has rapidly expanded the possibility of accurately monitoring organellar Ca\textsuperscript{2+} dynamics. The set up of a toolkit of aequorin-based probes targeted to the different subcompartments of chloroplasts (outer and inner envelope membranes, stroma, thylakoids) has allowed for the elucidation of stimulus-specific intra-organellar Ca\textsuperscript{2+} signals and their contribution to fine-tuning cytosolic Ca\textsuperscript{2+} signatures (Mehlner et al., 2012; Sello et al., 2016; Sello et al., 2018). A complementary approach based on the design of a cameleon probe directed to the chloroplast stroma further permitted Ca\textsuperscript{2+} imaging in single chloroplasts, highlighting organelle-autonomous Ca\textsuperscript{2+} transients (Loro et al., 2016). The establishment of aequorin reporters targeted to the thylakoid lumen and thylakoid membrane highlighted the ability of thylakoids to store 3- to 5-fold higher [Ca\textsuperscript{2+}] with respect to the stroma (about 500 nM in the thylakoid lumen versus 100-150 nM in the stroma, in resting conditions in the dark), as well as their contribution to the modulation of intra-chloroplast Ca\textsuperscript{2+} signals (Sello et al., 2018).

Chloroplast Ca\textsuperscript{2+} signals have been shown to be triggered by a large number of different stimuli of both biotic and abiotic nature. Elicitors of plant defence responses, such as the fungal-derived protein cryptogein and the plant cell wall-derived pectin fragments oligogalacturonides, were found to evoke transient Ca\textsuperscript{2+} elevations in the chloroplast stroma of *Nicotiana tabacum* and *Arabidopsis thaliana* plant cell suspension cultures (Manzoor et al., 2012; Sello et al., 2018). Moreover, the bacterial flagellin peptide flg22 was demonstrated to trigger a chloroplast Ca\textsuperscript{2+} response in the chloroplast stroma of *Arabidopsis rosette leaves*, peaking later than the cytosolic Ca\textsuperscript{2+} elevation (Nomura et al., 2012; Nomura and Shiina, 2014). In this latter work, a striking chloroplast-mediated transcriptional reprogramming during plant immune responses was demonstrated, uncovering an unanticipated link between chloroplast and nuclear plant innate immunity via ROS and Ca\textsuperscript{2+} signaling (Stael et al., 2015). The calcium-sensing receptor CAS, a thylakoid-localized protein of not yet well-defined function, was found to be involved in the generation of the flg22-induced stromal Ca\textsuperscript{2+} transient and chloroplast-mediated activation of defence gene expression (Nomura et al., 2012).

Different abiotic cues, such as cold, oxidative, salt and osmotic stresses were found to evoke stimulus-specific Ca\textsuperscript{2+} signals in the chloroplast stroma (Nomura et al., 2012; Sello et al., 2016; Sello et al., 2018; Teardo et al, 2019). Whereas these stimuli were shown to activate Ca\textsuperscript{2+} responses in both chloroplasts and non-green plastids (Sello et al., 2016), the light-to-dark transition was found to elicit a chloroplast-specific response (Sello et al., 2016; Loro et al., 2016). Although the precise mechanisms underlying dark-induced chloroplast Ca\textsuperscript{2+} fluxes remain to be unravelled, the circadian gating of dark-induced chloroplast and cytosolic Ca\textsuperscript{2+} elevations has recently been demonstrated (Martí Ruiz et al., 2020), uncovering an intriguing link between eukaryotic circadian clocks and chloroplasts.

In contrast to the above-mentioned stimuli, that have been demonstrated to trigger Ca\textsuperscript{2+} transients in both chloroplasts and the cytosol, increases in absolute temperature were found to evoke Ca\textsuperscript{2+} responses specific to chloroplasts, as no corresponding elevations were detected in the cytosol (Lenzoni and Knight, 2019). Interestingly, also in this case the chloroplast Ca\textsuperscript{2+} response was found to be partially dependent on CAS (Lenzoni and Knight, 2019).

Taken together, the above findings strongly highlight the ability of chloroplasts to perceive and transduce environmental signals in a Ca\textsuperscript{2+}-dependent manner. However, compared to the large amount of information progressively cumulating on the generation of chloroplast Ca\textsuperscript{2+} signals, information about Ca\textsuperscript{2+}-permeable channels/transporters localized at chloroplast membranes has long lagged behind.
CURRENT KNOWLEDGE OF THE MOLECULAR PLAYERS INVOLVED IN Ca\textsuperscript{2+} HANDLING IN CHLOROPLASTS

The extent, duration and frequency (i.e. signature) of free Ca\textsuperscript{2+} elevation in the cytosol ([Ca\textsuperscript{2+}]\textsubscript{cyt}) acts as a signal to be implemented in the transducing machinery of the cell. Different stimuli are followed by different Ca\textsuperscript{2+} signatures, leading in turn to different specific responses, in terms of gene expression, protein activity and localization. The Ca\textsuperscript{2+} signature is shaped by the activity of Ca\textsuperscript{2+}-permeable channels and transporters regulating the ion entry into and exit from the cytosol, respectively. Ca\textsuperscript{2+}-permeable channels are grouped in five families: cyclic nucleotide-gated channels (CNGCs), glutamate receptors-like channels (GLRs), two-pore channels (TPCs), mechanosensitive channels (MCAs), hyperosmolality gated channels (OSGAs) (Demidchik et al., 2018). Ca\textsuperscript{2+} transport off the cytosol to restore the resting [Ca\textsuperscript{2+}]\textsubscript{cyt} is mediated by energy-driven pumps/transporters belonging to the P-type ATPases, such as P1B-type calcium/heavy metal cation-transporting ATPase (AtHMA1), P2A-type calcium-transporting ATPase (ECAs) and P2B-type calcium-transporting ATPase (ACAs) (García Bossi et al., 2020). Other Ca\textsuperscript{2+} transporters are grouped in the CaCA family (Ca2+-type proton:cation exchanger, CCX-type cation:calcium cation exchanger, NCL/EF-CAX-type cation exchanger, EF-CAX-type cation exchanger) (Pittman and Hirschi, 2016) and CaCA2 family (PAM71-type manganese/calcium cation transporter).

The transduction of the Ca\textsuperscript{2+} signal is mediated by Ca\textsuperscript{2+}-dependent/binding proteins. The Arabidopsis genome encodes for 250 proteins harbouring at least one Ca\textsuperscript{2+} binding domain (EF-hand), hence acting as putative Ca\textsuperscript{2+} sensors [e.g. (Ranty et al., 2016)]. Calmodulins (CaMs), calmodulin-like (CaMLs), calcineurin B-like proteins (CBLs) and Ca\textsuperscript{2+}-dependent protein kinases (CPKs) all harbour EF hand motifs. Ca\textsuperscript{2+} sensors directly (CPKs) or indirectly (CaMs, CaMLs, CBLs) [e.g. (Sanyal et al., 2015; Kudla et al., 2018)] modulate protein activity (e.g. ion channels, metabolic enzymes) and/or protein subcellular localization (e.g. transcription factors). The redundancy of sensor isoforms allows the discrimination between different signals and carry the specificity of the message brought by the Ca\textsuperscript{2+} signature.

To our knowledge, Ca\textsuperscript{2+}-binding proteins acting as buffers in the chloroplast have not yet been identified. Nevertheless, organellar Ca\textsuperscript{2+} buffering mechanisms are likely to play an essential role, generating heterogeneity in local Ca\textsuperscript{2+} concentrations inside chloroplasts. How Ca\textsuperscript{2+} is stored in the chloroplast remains an open question for future investigations, aimed to unravel whether Ca\textsuperscript{2+} interacts with specific Ca\textsuperscript{2+} binding proteins or with the thylakoid surface, which harbours a significant amount of phosphorylated proteins that have been suggested to bind calcium ions (Rocha and Vothknecht, 2012; Stael et al., 2012a; Stael et al., 2012b).

The major part of research carried out so far has focused on the analysis of the cytosolic Ca\textsuperscript{2+} signature, but the possibility to study Ca\textsuperscript{2+} dynamics in organelles by targeting Ca\textsuperscript{2+} probes to plastids has recently allowed the understanding of the existence of organellar Ca\textsuperscript{2+} transients in response to external stimuli. These findings pose the question of the identity of players involved in shaping and transducing the Ca\textsuperscript{2+} signal coming from organelles. The existence of peculiar and dedicated pathways for Ca\textsuperscript{2+} handling in organelles can be a possibility, and/or the machinery may comprise some already known players that may localize to chloroplasts as well (Finazzi et al., 2015; Pottosin and Shabala, 2015; Carraretto et al., 2016).

Recently, two proteins belonging to the family of the mitochondrial calcium uniporter (MCU) have been found to mediate Ca\textsuperscript{2+} transport across the mitochondria and chloroplast membranes, respectively AtMCU1 (Teardo et al., 2017) and AtMCU6 (later renamed AtcMCU) (Teardo et al., 2019). In animal cells the only isoform, MCU (De Stefani et al., 2011; Baughman, 2011) is responsible for Ca\textsuperscript{2+} loading into mitochondria, thus helping recovery of resting [Ca\textsuperscript{2+}]\textsubscript{cyt}. New evidence supports the involvement of MCU isoforms in shaping the organellar Ca\textsuperscript{2+} signatures in plants as well (Wagner et al., 2015; Teardo et al., 2017; Selles et al., 2018; Teardo et al., 2019).

In particular, cMCU is involved in the generation of the stromal Ca\textsuperscript{2+} transient specific for the osmotic stress and mutants lacking cMCU showed an improved drought tolerance (Stael, 2019; Teardo et al., 2019).

It is now commonly acknowledged that a protein can localize to different cell compartments (Karnieli and Pines, 2005), as it has been proven also for proteins involved in Ca\textsuperscript{2+} handling (Table 1). AtGLR3.4 and AtGLR3.5, two Ca\textsuperscript{2+} -permeable channels belonging to the GLR family, have a dual localization, at the plasma membrane and chloroplasts the former (Teardo et al., 2010; Teardo et al., 2011), in mitochondria and chloroplasts the latter (Teardo et al., 2015). Both seem to play a role in ABA signaling under abiotic stress (Cheng et al., 2018; Ju et al., 2020), although their direct involvement in organellar Ca\textsuperscript{2+} signaling under abiotic stress has to be investigated more in depth.

Querying the protein databases Uniprot (The UniProt Consortium, 2019), SUBA4 (Hooper et al., 2017) and Aramemnon (Schwacke et al., 2003) for A. thaliana records with plastidal localization and using “calcium” as keyword, 682 hits can be found in SUBA4, only 43 in Aramemnon and 42 in Uniprot. Table 1 shows all those proteins belonging to the above-mentioned classes of channels/transporters, sensors and kinases involved in Ca\textsuperscript{2+} signature formation and signaling, whose plastidial localization has been predicted or demonstrated by MS/MS or by fusion to fluorescent proteins (FP).

23 out of 47 proteins belong to Ca\textsuperscript{2+} channels/transporters; 6 are confirmed to be located in plastid membranes either by biochemical and cell biology methods or by mass spectrometry. Among them, for AtcMCU, AtGLR3.4 and AtGLR3.5 a role in stress response was suggested. Altogether, these channels/transporters can be involved in the formation of the plastidial Ca\textsuperscript{2+} transients, along with the putative calcium-transporting protein PAM71/BICAT (Frank et al., 2019). However, this latter protein seems to play a prevalent role in manganese homeostasis rather than in calcium homeostasis (Schneider et al., 2016;
| Gene ID | Protein Name | Description | Protein family | Predicted Localization (Aramemnon or SUBA4) | Experimental Localization (FP, MS/MS) | involved in | references |
|---------|--------------|-------------|----------------|---------------------------------|---------------------------------|-------------|------------|
| **Ca²⁺ sensors** | | | | | | |
| At1g18890 | AtCPK10 | Calcium-dependent protein kinase 10 | Calcium Dependent Protein Kinase | plastid, mitochondrion, cytosol, nucleus | nucleus | drought, ABA, stomatal closure | Zou et al., 2010; Liu et al., 2017 |
| At1g35670 | AtCPK11 | Calcium-dependent protein kinase 11 | Calcium Dependent Protein Kinase | plastid, mitochondrion, cytosol, nucleus | nucleus, cytosol, PM | pollen tube growth, salt and drought induced, salt and ABA signaling | Urao et al., 1994; Rodriguez Milla et al., 2006; Zhu et al., 2007; Benschop et al., 2007; Ito et al., 2011; Zhao et al., 2013 |
| At2g17890 | AtCPK16 | Calcium-dependent protein kinase 16 | Calcium Dependent Protein Kinase | plastid, mitochondrion, cytosol | PM | | |
| At2g31500 | AtCPK24 | Calcium-dependent protein kinase 24 | Calcium Dependent Protein Kinase | plastid, mitochondrion, cytosol, nucleus | nucleus, PM | pollen tube growth | Gutermuth et al., 2013; Zhao et al., 2013 |
| At2g38910 | AtCPK20 | Calcium-dependent protein kinase 20 | Calcium Dependent Protein Kinase | plastid, nucleus, membrane | plastid, PM | | |
| At3g10660 | AtCPK18 | Calcium-dependent protein kinase 2 | Calcium Dependent Protein Kinase | plastid, nucleus, mitochondrion, cytosol | PM | | |
| At4g04695 | AtCPK31 | Calcium-dependent protein kinase 31 | Calcium Dependent Protein Kinase | nucleus, plastid, mitochondrion, cytosol | plastid, PM | arsenite uptake | Helm et al., 2014; Ji et al., 2017 |
| At4g04720 | AtCPK21 | Calcium-dependent protein kinase 21 | Calcium Dependent Protein Kinase | PM, cytosol, mitochondrion, plastid, nucleus | PM | interacts with SLAC1, AB1, SLAH3, GORK | |
| At4g09570 | AtCPK4 | Calcium-dependent protein kinase 4 | Calcium Dependent Protein Kinase | cytosol, nucleus, mitochondrion, plastid | PM, cytosol, nucleus | ABA and salt response; interacts with plastid proteins | Dammann et al., 2003; Zhu et al., 2007; Mitra et al., 2009; Uno et al., 2009; Ito et al., 2011; Li et al., 2018 |
| At4g21940 | AtCPK15 | Calcium-dependent protein kinase 15 | Calcium Dependent Protein Kinase | cytosol, plastid, nucleus, mitochondrion | PM | stomatal closure | Dammann et al., 2003; Alexandersson et al., 2004; Nelson et al., 2006; Benschop et al., 2007; Marmagne et al., 2007; Mitra et al., 2009; Keinath et al., 2010; Geiger et al., 2010; Zhang and Peck, 2011; Elmore et al., 2012; Nikolovski et al., 2012; Bernfur et al., 2013; Demir et al., 2013; Zargar et al., 2015; De Michele et al., 2016; van Kleeff et al., 2018 |
| At4g23650 | AtCPK3 | Calcium-dependent protein kinase 3 | Calcium Dependent Protein Kinase | plastid, mitochondrion, cytosol, nucleus | cytosol, nucleus, PM, Golgi, tonoplast | | |
| At4g36070 | AtCPK18 | Calcium-dependent protein kinase 18 | Calcium Dependent Protein Kinase | plastid, mitochondrion, peroxisome, PM | | | |
| At5g04870 | AtCPK17 | Calcium-dependent protein kinase 1 | Calcium Dependent Protein Kinase | plastid, nucleus, cytosol, mitochondrion | peroxisome, MVB, cytosol, PM | salt and drought | Dammann et al., 2003; Chen et al., 2010; Drakakaki et al., 2012; De Michele et al., 2016; Huang et al., 2018 |
| At5g12180 | AtCPK17 | Calcium-dependent protein kinase 17 | Calcium Dependent Protein Kinase | cytosol, nucleus, mitochondrion, plastid | PM | pollen tube tip growth | Myers et al., 2009; Gutermuth et al., 2013; Bernfur et al., 2013 |

(Continued)
| Gene ID   | Protein Name | Description              | Protein family                  | Predicted Localization (Aramemnon or SUBA4) | Experimental Localization (FP, MS/MS) | involved in                                                                 | references                                                                 |
|----------|--------------|--------------------------|---------------------------------|---------------------------------------------|---------------------------------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| At5g12480 | AtCPK7       | Calcium-dependent protein kinase 7 | Calcium Dependent Protein Kinase | plastid, mitochondrion, cytosol, nucleus    | PM, Golgi                            | root hydraulic conductivity                                                      | Dammann et al., 2003; Marmagne et al., 2007; Benschop et al., 2007; Elmore et al., 2012; Heard et al., 2015; Li et al., 2015 |
| At5g19360 | AtCPK34      | Calcium-dependent protein kinase 34 | Calcium Dependent Protein Kinase | cytosol, nucleus, mitochondrion, plastid    | PM                                    | pollen tube tip growth                                                           | Myers et al., 2009; Gutermuth et al., 2013; Ber_nf_ et al., 2013 |
| At5g19450 | AtCPK8       | Calcium-dependent protein kinase 8 | Calcium Dependent Protein Kinase | cytosol, nucleus, mitochondrion, plastid    | PM                                    | ABA signaling and H₂O₂ homeostasis in guard cells                               | Dammann et al., 2003; Nühse et al., 2003; Nühse et al., 2004; Benschop et al., 2007; Chen et al., 2010; Keinath et al., 2010; Zhang and Peck, 2011; Elmore et al., 2012; Zargar et al., 2015; Zou et al., 2015 Benschop et al., 2007; Marmagne et al., 2007; Chen et al., 2010; Keinath et al., 2010; Zhang and Peck, 2011; Li et al., 2012; Szymanski et al., 2015; De Michele et al., 2016 |
| At5g24430 | AtCRK4       | Calcium-dependent protein kinase 4 | Calcium Dependent Protein Kinase | plastid, nucleus, cytosol, mitochondrion    | PM                                    |                                                                                |                                                                                |
| At5g66210 | AtCPK28      | Calcium-dependent protein kinase 28 | Calcium Dependent Protein Kinase | cytosol, plastid, mitochondrion, nucleus    | PM                                    | plant immunity                                                                  | Dammann et al., 2003; Benschop et al., 2007; Elmore et al., 2012; Monaghan et al., 2014; Monaghan et al., 2015; Matschi et al., 2015; De Michele et al., 2016 |
| At2g15680 | AtCML30      | Calmodulin-like protein 30   | Calmodulin-like protein         | plastid, mitochondrion, cytosol, PM         | mitochondrial                         |                                                                                | Chigri et al., 2012                                                                 |
| At2g41410 | AtCML35      | Probable calcium-binding protein CML35 | Calmodulin-like protein         | plastid, mitochondrion, cytosol, PM         | PM, vacuole                          | dark induced                                                                    | Lee et al., 2005; Benschop et al., 2007; Whiteman et al., 2008; Elmore et al., 2012; Li et al., 2012; De Michele et al., 2016 |
| At2g43290 | AtCML5       | Calmodulin-like protein 5    | Calmodulin-like protein         | plastid, mitochondrion, cytosol, PM, ER     | ER, Golgi                            | dark and touch induced                                                          | Lee et al., 2005; Ruge et al., 2016                                                                 |
| At3g10190 | AtCML36      | Calmodulin-like protein 36   | Calmodulin-like protein         | plastid, nucleus, cytosol, PM, extracellular plastid, nucleus, mitochondrion, cytosol, PM, ER                                                                                     | PM                                    | ACA8 activation                                                                 | Benschop et al., 2007; Astegno et al., 2017                                                                 |
| At3g29000 | AtCML45      | Calmodulin-like protein 45   | Calmodulin-like protein         | plastid, mitochondrion, cytosol, PM         | PM                                    |                                                                                |                                                                                |
| At3g50770 | AtCML41      | Probable calcium-binding protein CML41 | Calmodulin-like protein         | plastid, mitochondrion, cytosol, PM         | PM, vacuole                          | drought, wounding                                                              | Vanderbeld and Snedden, 2007; Inzé et al., 2012; Scholz et al., 2014; Scholz et al., 2015 Wagner et al., 2015; Teardo et al., 2017 |
| At4g26470 | AtCML21      | Calmodulin-like protein 21   | Calmodulin-like protein         | cytosol, PM, mitochondrion, nucleus, plastid | cell wall                            |                                                                                | Nguyen-Kim et al., 2016                                                                 |
| At5g04170 | AtCML50      | Probable calcium-binding protein CML50 | Calmodulin-like protein         | cytosol, PM, mitochondrion, nucleus, plastid | cell wall                            |                                                                                | Nguyen-Kim et al., 2016                                                                 |
| At5g39670 | AtCML46      | Calmodulin-like protein 46   | Calmodulin-like protein         | cytosol, plastid, ER, Golgi, nucleus, extracellular plastid, nucleus, mitochondrion, cytosol, PM, ER                                                                                  | PM                                    |                                                                                |                                                                                |
| At5g42380 | AtCML37      | Calcium-binding protein CML37 | Calmodulin-like protein         | cytosol, nucleus                            | PM                                    | drought, wounding                                                              | Vanderbeld and Snedden, 2007; Inzé et al., 2012; Scholz et al., 2014; Scholz et al., 2015 Wagner et al., 2015; Teardo et al., 2017 |
| At4g32060 | AtMcUC       | Calcium uptake protein, mitochondrial | Calmodulin-like protein         | PM, mitochondrion, plastid                  | PM                                    | regulation of Ca²⁺ unporters (MCUs)                                                 |                                                                                |
| At4g33000 | AtCBL10      | Calcineurin B-like protein 10 | Calmodulin-like protein         | plastid, mitochondrion, PM, ER              | PM, tonoplast                        | salt tolerance                                                                  | Mitra et al., 2009; Ma et al., 2019; Yang et al., 2019                                                                 |

(Continued)
| Gene ID | Protein Name | Description | Protein family | Predicted Localization (Aramemnon or SUBA4) | Experimental Localization (FP, MS/MS) | involved in | references |
|---------|--------------|-------------|----------------|-----------------------------------------------|--------------------------------------|-------------|------------|
| **At5g23060** | AtCAS | Calcium sensing receptor | Calcium sensing receptor | plastid, mitochondrion | plastid, thylakoid, Golgi, mitochondrion, nucleus | high light, stomatal regulation, drought tolerance | Vainonen et al., 2008; Weinl et al., 2008; Behnens et al., 2013; Hein et al., 2014; Tomizoi et al., 2014; Wang et al., 2014; Heard et al., 2015; Fakhi et al., 2016; Fromm et al., 2016; Melonek et al., 2016; Senkler et al., 2017; Cutolo et al., 2019 |
| **Ca²⁺ transporters/channels** | | | | | |
| **At1g53210** | AtNCL | Sodium/calcium exchanger | NCL/EF-CAX-type cation exchanger | plastid, mitochondrion, Golgi, cytosol, PM, ER | PM, tonoplast | flowering time, auxin signaling, salt stress | Nikolovski et al., 2012; Elmore et al., 2012; Li et al., 2016; Wang et al., 2012; Yoshida et al., 2013; Szymanski et al., 2015; Zargar et al., 2015; Li et al., 2016 |
| **At2g34020** | | | EF-CAX-type cation exchanger | plastid, mitochondrion, Golgi, PM, tonoplast | | | |
| **At2g38170** | AtCAX1 | High-affinity calcium/proton cation exchanger | CAX-type proton:calcium cation exchanger | plastid, mitochondrion, Golgi, PM, tonoplast | | Cd²⁺ tolerance; pH regulation; hormone signaling; guard cell dynamics; stress response | Cheng et al., 2003; Conn et al., 2011; Cho et al., 2012; Ballardini et al., 2015; Hocking et al., 2017 |
| **At3g14070** | AtCCX3/ CAX9 | Cation/calcium exchanger 3 | CXX-type cation:cation exchanger | plastid, mitochondrion, Golgi, PM, ER | endomembrane | | Morris et al., 2008 |
| **At3g51860** | AtCAX3 | High-affinity calcium/proton cation exchanger | CAX-type cation:cation exchanger | plastid, mitochondrion, Golgi, PM, tonoplast | tonoplast | pH regulation; hormone signaling; guard cell dynamics | Manohar et al., 2011; Cho et al., 2012; Hocking et al., 2017 |
| **At5g01490** | AtCAX4 | High-affinity calcium/proton cation exchanger | CAX-type cation:cation exchanger | plastid, ER, PM, tonoplast | tonoplast | Cd²⁺ accumulation | Cheng et al., 2002; Mei et al., 2009 |
| **At2g23790** | AtMCU3 | Putative channel component of MCU calcium uniporter complex | Component of MCU calcium uniporter complex | plastid, mitochondrion, nucleus | tonoplast | | Yoshida et al., 2013 |
| **At4g36820** | AtMCU4 | Putative channel component of MCU calcium uniporter complex | Component of MCU calcium uniporter complex | mitochondrion, chloroplast, nucleus | mitochondrion | | Teardo et al., 2017 |
| **At5g66650** | AtMCU6/ AtcMCU | Putative channel component of MCU calcium uniporter complex | Component of MCU calcium uniporter complex | plastid, mitochondrion, nucleus | plastid, mitochondrion | drought, hypoxia | Teardo et al., 2019; Lee and Bailey-Serres, 2019 |
| **At1g05200** | AtGLR3.4 | Putative GLR-type amino acid-gated calcium cation channel | GLR-type ligand-gated cation channel | PM, plastid, ER, Golgi, mitochondrion | plastid, PM | Ca²⁺ transport; salt and cold stress; ABA signaling; seed germination; lateral root development stomatal closure | Meyerhoff et al., 2005; Stephens et al., 2008; Teardo et al., 2011; Vincill et al., 2013; Cheng et al., 2018 |
| **At2g17260** | AtGLR3.1 | Putative GLR-type channel permeable calcium cation channel | GLR-type ligand-gated cation channel | PM, plastid, ER, Golgi | endomembrane | | Cho et al., 2009; Kong et al., 2016; Nguyen et al., 2018 |
| **At2g32390** | AtGLR3.5 | Putative GLR-type channel permeable calcium cation channel | GLR-type ligand-gated cation channel | PM, plastid, mitochondrion, nucleus | plastid, mitochondrion | Ca²⁺ transport; ABA signaling; seed germination; stomatal closure | Teardo et al., 2015; Kong et al., 2016; Ju et al., 2020 |

(Continued)
| Gene ID  | Protein Name | Description | Protein family | Predicted Localization (Aramemnon or SUBA4) | Experimental Localization (FP, MS/MS) | involved in | references |
|---------|--------------|-------------|----------------|---------------------------------------------|--------------------------------------|-------------|------------|
| At5g11210 | AtGLR2.5 | Putative GLR-type calcium cation-permeable channel | GLR-type ligand-gated cation channel | plastid, mitochondrion, PM | PM | Mitra et al., 2009 |
| At1g69450 | AtOSCA2.4 | Early-responsive to dehydration stress protein (ERD4) | OSCA1/2/3-type Ca^{2+}-permeable hyperosmolality-gated channel | chloroplast, mitochondrion, PM, Golgi | PM | Yuan et al., 2014 |
| At3g54510 | AtOSCA2.5 | Hyperosmolality-gated calcium-permeable channel | OSCA1/2/3-type Ca^{2+}-permeable hyperosmolality-gated channel | mitochondrial, plastid, nucleus, Golgi,ER, PM | ER, mitochondrion, plastid | Lee et al., 2011 |
| At4g02900 | AtOSCA1.7 | Hyperosmolality-gated calcium-permeable channel | OSCA1/2/3-type Ca^{2+}-permeable hyperosmolality-gated channel | mitochondrial, plastid, nucleus, Golgi,ER, PM | ER, mitochondrion, plastid | Lee et al., 2011 |
| At4g35870 | AtOSCA4.1/AtGFS10 | Calcium-permeable channel-like protein | OSCA4-type unspecified channel | chloroplast, mitochondrion, PM, Golgi, nucleus | Golgi | Heard et al., 2015 |
| At4g37270 | AtHMA1 | Thapsigargin-sensitive calcium/heavy metal cation transporting P1B-type ATPase | P1B-type heavy metal cation transporting ATPase | plastid, mitochondrial, PM | chloroplast envelope | photosynthesis | Seigneurin-Berny et al., 2006; Higuchi et al., 2009; Ferro et al., 2010; Nikolovski et al., 2012; Tomizioli et al., 2014 |
| At1g27770 | AtACA1 | Calcium-transporting ATPase | P2B-type calcium cation transporting ATPase | plasma membrane, plastid, cytosol, ER, mitochondrion, nucleus | plastid, ER, PM, tonoplast, microtubule | Huang et al., 1993; Dunkley et al., 2006; Benschop et al., 2007; Mitra et al., 2009; Zhang and Peck, 2011; Yoshida et al., 2013; Hamada et al., 2013 |
| At3g21180 | AtACA9 | Calcium-transporting ATPase | P2B-type calcium cation transporting ATPase | plasma membrane, plastid, cytosol, ER, mitochondrion, nucleus | plasma membrane, plastid, cytosol, ER, mitochondrion, nucleus | pollen development, salt tolerance in yeast | Schiott et al., 2004; Tomizioli et al., 2014 |
| At4g37640 | AtACA2 | Calcium-transporting ATPase | P2B-type calcium cation transporting ATPase | PM, ER, plastid, mitochondrial, vacuole | Golgi, ER, PM | Dunkley et al., 2006; Benschop et al., 2007; Anil et al., 2008; Zhang and Peck, 2011; Nikolovski et al., 2012; Heard et al., 2015; Tomizioli et al., 2014 |
| At5g53010 | AtACA1 | Calcium-transporting ATPase, putative | P2B-type calcium cation transporting ATPase | mitochondrial, PM, ER plastid | plastid | Wang et al., 2016; Schneider et al., 2016; Frank et al., 2019 |
| At1g64150 | AtBICAT1/AtPAM71/AtCCHA1 | Putative calcium/manganese cation transporter | PAM71-type manganese/calcium cation transporter | plastid, mitochondrial thylakoid membrane | Mn^{2+} homeostasis, phototropic growth, chloroplast Ca^{2+} homeostasis, photosynthesis | Ferro et al., 2010; Zybailov et al., 2008; Ferro et al., 2010; Tomizioli et al., 2014; Eisenhut et al., 2018; Zhang et al., 2018; Frank et al., 2019 |
| At4g13590 | AtBICAT2/AtCMT1 | Putative calcium/manganese cation transporter | PAM71-type manganese/calcium cation transporter | plastid, mitochondrial chloroplast envelope | Mn^{2+} homeostasis, phototropic growth, chloroplast Ca^{2+} homeostasis, photosynthesis | Ferro et al., 2010; Zybailov et al., 2008; Ferro et al., 2010; Tomizioli et al., 2014; Eisenhut et al., 2018; Zhang et al., 2018; Frank et al., 2019 |
| Others | At1g64850 | Calcium-binding EF hand family protein | | vacuole, mitochondrial, plastid, nucleus, vacuole | plastid, peroxisome | Reumann et al., 2009; Ferro et al., 2010; Nikolovski et al., 2012 |
Zhang et al., 2018). In addition to Ca\textsuperscript{2+} channels and transporters, Ca\textsuperscript{2+} sensors, namely 21 proteins, are predicted to be located in plastids. However, only three have been confirmed so far: AtCPK20, AtCPK31, and AtCAS. It is worth to mention that CPK20, besides the plastidal localization that was confirmed by MS/MS approaches (Behrens et al., 2013), showed a plasma membrane localization when fused to reporter genes or co-expressed with other CPK members (Gutermuth et al., 2013). CPK31 has also been shown to localize at the plasma membrane when interacting with the arsenite transporter NIP1;1 (Ji et al., 2017). In addition, localization of many CPKs with chloroplast-targeting sequence can be affected by N-acylation. For example, AtCPK20 and 31 are located in the chloroplast, only if its N-terminus is unacylated (Stael et al., 2011). Interestingly, AtGRF9, a Ca\textsuperscript{2+}-regulated 14-3-3 protein, although not predicted to be located in chloroplasts, has been demonstrated to be present in many compartments, including plastids. This regulatory protein is involved in root and leaf development under water stress (He et al., 2015) and leaf development in general (Omidbakhshfard et al., 2012). In addition, localization of many CPKs with chloroplast-targeting sequence can be affected by N-acylation. For example, AtCPK20 and 31 are located in the chloroplast, only if its N-terminus is unacylated (Stael et al., 2011). Interestingly, AtGRF9, a Ca\textsuperscript{2+}-regulated 14-3-3 protein, although not predicted to be located in chloroplasts, has been demonstrated to be present in many compartments, including plastids. This regulatory protein is involved in root and leaf development under water stress (He et al., 2015) and leaf development in general (Omidbakhshfard et al., 2012), but its role in chloroplasts has not yet been explored.

The presence of members of protein families involved in Ca\textsuperscript{2+} transport/sensing supports the idea of a core-machinery determining the observed Ca\textsuperscript{2+} transients in the chloroplast stroma, and putatively in the thylakoid lumen as well. Ca\textsuperscript{2+} sensors are indeed present in plastids, although their activity in deciphering organelar Ca\textsuperscript{2+} signatures has not been fully demonstrated so far. Nevertheless, a recent work points to CAS as mediator of light response and photoacclimation (Cutolo et al., 2019).

The multiple localizations shown by some proteins in Table 1 awaits further investigation. Recent evidence is pointing to the hypothesis of an inter-connection between organelles and nucleus for material exchanging or signal propagation (Kmiecik et al., 2016). The presence of the Ca\textsuperscript{2+} handling machinery in multiple positions can be part of the retrograde signaling in response to adverse environmental conditions (Pornsiriwong et al., 2017).

### Structural and Functional Comparison between MCU Isoforms from Different Organisms and the Chloroplast-Localized Homologue in Plants

As mentioned above, AtMCU is one of the very few molecular entities among the plastidial Ca\textsuperscript{2+} channels/transporters shown to work as a Ca\textsuperscript{2+}-permeable ion channel, to mediate indeed Ca\textsuperscript{2+} flux across chloroplast envelope and to participate in the drought stress response in Arabidopsis. While many organisms have only one MCU isoform (Bick et al., 2012), Arabidopsis harbours 6 different isoforms: 5 with clear predicted subcellular localization to mitochondria, whereas AtMCU6/At5g66650 has a predicted localization to either plastoplasts and/or to mitochondria. Localization prediction was confirmed for AtMCU1/At1g09575 (Tearo et al., 2017), AtMCU2/At1g57610 (Wagner et al., 2015; Selles et al., 2018), AtMCU3/At2g23790 (Carraretto et al., 2016). For AtMCU6 an interesting situation was observed: in tissues harbouring mature chloroplasts, AtMCU6 was efficiently targeted to these photosynthetic organelles, whereas in roots the protein was found in mitochondria (Tearo et al., 2019). Thus, either plastid-specific partners promote targeting of AtMCU6/AtMCU1 targeting or depends on the metabolic state of a given cell. However, among the possible partners (https://string-db.org/network/3702.AT5G66650.1) no proteins with unique localization to chloroplasts are present. Thus, the mechanism by which dual localization occurs awaits clarification.

The N-terminal domain (NTD) of AtcMCU harbours motifs rich in acidic residues, one of which (107-118) playing a role in Ca\textsuperscript{2+} uptake by cMCU, as demonstrated by mutagenesis studies (D107A/E118K mutant) and Ca\textsuperscript{2+} uptake assays in an aequorin-based E. coli system (Tearo et al., 2019). Two groups independently set up the same system to study MCU activity,
namely that exploiting *E. coli* stably expressing aequorin (Teardo et al., 2019) or the fluorescent Ca\(^{2+}\) reporter GCaMP2 (Fan et al., 2018). This valuable tool allows a quick screening of the effect of MCU residues’ mutations and of chemical modulators on the Ca\(^{2+}\) flux-mediating activity and may become a method of choice for further structure-function studies.

One common feature of MCU homologs from fungi and Arabidopsis is that they can function as Ca\(^{2+}\)-permeable channels on their own in contrast to vertebrates, where the uniporter is a complex (MCUC) consisting of multiple subunits, including: 1) the channel forming unit (MCU) with two transmembrane segments and a conserved DXXE sequence forming the Ca\(^{2+}\) selectivity filter (see Figure 1); 2) regulatory EF-hand proteins MICU1-3; 3) a small, single-pass transmembrane protein, EMRE (Essential MCU REgulator) [for review see e.g. (Wagner et al., 2016)]. The structure of MCU homologs from various organisms has been recently solved: 1) from *Fusarium graminearum* and *Metarhizium acridum* revealing a dimer assembly of MCU (Fan et al., 2018); 2) from *Neurospora crassa* (Yoo et al., 2018); 3) from *Neosartorya fischeri* (Nguyen et al., 2018b); and from 4) zebrafish and *Cyphellophora europaea* (Baradaran et al., 2018). All these homologues share high sequence similarity in their transmembrane domains, show a similar pore architecture and a high structural similarity of the NTDs (despite relatively low sequence homology). The amino acid sequence is more similar between Arabidopsis and *Dictyostelium discoideum* than between AtMCUs and human MCU (Teardo et al., 2017). This similarity apparently translates also to the tertiary structure of the two proteins, at least regarding the N-terminal domain, whose structure has been recently resolved for Dictyostelium MCU, proving its divergent evolution (doi: https://doi.org/10.1101/848002) (see Figure 1).

In plants and fungi, the pore-forming unit MCU alone is able to allow Ca\(^{2+}\) flux, without the need of EMRE, as confirmed by different groups (Tsai et al., 2016; Teardo et al., 2017; Fan et al., 2018; Teardo et al., 2019). In fact, homologs of EMRE are not present in these organisms. The cryo-EM structure of the human MCU-EMRE complex (Wang et al., 2019) suggests that NTD mediates the dimerization of two human MCU tetramers, thereby modulating the function of the channel [although deletion of NTD does not affect Ca\(^{2+}\) flux (Lee et al., 2015)]. In contrast to other MCUs, an (R/K)/Q/(R/K/D)/K/L motif is found in the L2 (Oxenoid et al., 2016) (now called CC2a for coiled-coiled domain 2a) (Wang et al., 2019) region of Arabidopsis, Dictyostelium and NfMCU (Teardo et al., 2017; Wang et al., 2019), all being able to form functional MCU without EMRE. It has been proposed that the extended side chain of HsMCU R297 (missing in the above MCUs) on CC2a connects the gate-forming juxtamembrane loop (JML) of MCU to EMRE by forming hydrogen bonds with the hydroxyl group of highly conserved T285 (on the JML of MCU) and a valine residue of EMRE. Interaction between CC2a and EMRE has been proposed as a crucial factor determining the conductivity of the channel formed by MCU tetramers. On the other hand, in the EMRE-independent Dictyostelium MCU, deletion of either CC1 or CC2
caused the loss of function of MCU (Yamamoto et al., 2019), suggesting that these two domains are crucial for MCU function independently of their ability to bind EMRE. Altogether, determination of structural differences among various MCUs accounting for the requirement of EMRE for channel function requires further work.

CONCLUSIONS AND PERSPECTIVES

In these last few years there has been a surge of papers on Ca\(^{2+}\) signaling in chloroplasts, witnessing the crucial role increasingly attributed to these plant-unique organelles in the orchestration of the complex Ca\(^{2+}\) signaling network of the plant cell. We foresee that the newly available experimental tools to investigate the role of thylakoids in Ca\(^{2+}\)-mediated signal transduction, the molecular identification of Ca\(^{2+}\) channels/transporters in chloroplast membranes and the determination of the structure of transmembrane proteins by cryo-EM will lead to a rapid development of this exciting field of plant research. Future plant organelar Ca\(^{2+}\) signaling studies should also focus on non-photosynthetic plastids, which have recently been proposed to trigger tissue-specific signaling involved in mounting plant systemic stress response (Beltran et al., 2018). Furthermore, the potential interplay of chloroplasts with other intracellular Ca\(^{2+}\)-mobilizable stores should also be taken into consideration, in view of the well-known structural and functional interactions established by plastids with other organelles (Mathur et al., 2012).

AUTHOR CONTRIBUTIONS

LN, EF, and IS jointly contributed to the writing of this manuscript. LC designed the structural model of cMCU presented in Figure 1. All authors reviewed and approved the final version of the submitted manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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