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INTEGRATION OF PLASTIDS IN THE PLANT CALCIUM SIGNALLING NETWORK IN RESPONSE TO ENVIRONMENTAL STIMULI

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SUMMARY

This thesis reports the results that I have obtained during my three-year PhD course in Biosciences and Biotechnologies, *curriculum* Evolutionary Biology.

My research activity aimed to elucidate how plastids, a diverse family of organelles found ubiquitously in plants and various algae, are involved in Ca^{2+} homeostasis and Ca^{2+}-based signal transduction events in response to environmental stimuli in the model plant *Arabidopsis thaliana*.

In plants, Ca^{2+} is used as an intracellular messenger to transduce a wide variety of abiotic and biotic stimuli, that evoke specific intracellular spatio-temporal Ca^{2+} signals. The differential Ca^{2+} signatures are further decoded by Ca^{2+} sensor proteins into stimulus-specific metabolic and transcriptional responses.

Regarding the internal compartmentalization of Ca^{2+}, most of the studies have been focused so far on the vacuole, that contains a high free Ca^{2+} concentration ($[Ca^{2+}]$), and for which extensive knowledge is already available about Ca^{2+} transporters localized at the tonoplast. Nevertheless, increasing evidence suggest that additional intracellular compartments may play some role as Ca^{2+} mobilizable stores, such as the endoplasmic reticulum, mitochondria and chloroplasts.

Concerning chloroplasts, it has long been known that Ca^{2+} is important in the organellar physiology, as it is involved in the modulation of photosynthesis, as well as in other plastid-localized processes, such as organelle division and the import of nuclear-encoded proteins. Nevertheless, the role of Ca^{2+} in chloroplasts, and in plastids in general, is still elusive, because only little information is so far available on the involvement of plastids in Ca^{2+} homeostasis and on the generation of specific Ca^{2+} signals inside plastids. In order to better understand the integration of plastids in the Ca^{2+}-mediated signalling pathways of the plant cell, it is essential to monitor Ca^{2+} dynamics inside the organelle in a sensitive and accurate way and to identify the Ca^{2+} mobilization mechanisms located at plastid membranes.

During my PhD activity, *Arabidopsis thaliana* cell suspension cultures stably expressing the bioluminescent Ca^{2+} reporter aequorin targeted to different chloroplast subcompartments, *i.e.* the surface of the outer envelope, the stroma, the thylakoid membrane and the thylakoid lumen, were set up. *Arabidopsis* plants stably transformed with the constructs encoding the different chloroplast-targeted aequorin chimeras were

made available for this project by Prof U. Vothknecht (Munich, Germany) in the framework of a collaboration between our two laboratories.

As a first step, heterotrophic cell suspension cultures, containing amyloplasts as functional types of plastids, and stably expressing aequorin in the cytosol, plastid outer envelope and stroma, respectively, were challenged with abiotic and biotic stimuli whose Ca^{2+}-mediated signal transduction is well established, such as oxidative stress, salinity, drought, cold shock and oligogalacturonides (pectic fragments of the plant cell wall released during pathogen attack). Aequorin-based Ca^{2+} measurements demonstrated the occurrence of stimulus-specific Ca^{2+} changes characterized by different kinetic parameters in each subcellular location. Moreover, the comparison of the Ca^{2+} traces indicated an essential role played by plastids in switching off different cytosolic Ca^{2+} signals. The set-up of both heterotrophic and autotrophic cell suspension cultures stably expressing aequorin targeted to the stroma allowed for a dissection of the different contribution of amyloplasts and chloroplasts, two different functional types of plastids, in the organellar Ca^{2+} dynamics in response to environmental signals. Although the Ca^{2+} responses of non-green plastids and dark-adapted chloroplasts were found to be very similar, significant differences in the amplitude of specific plastidial Ca^{2+} changes (*i.e.* to salinity and drought) emerged when the photosynthetic activity of chloroplasts was reactivated by light. Peculiarities in Ca^{2+} responses to environmental stimuli suggest that different functional types of plastids might cover different roles in various organs of the plant in terms of adaptation to changes in environmental conditions, especially when these organelles are in a particular metabolic status **[Chapter 3]**.

Information about plastid Ca^{2+} dynamics in response to environmental stimuli was subsequently complemented by the set-up of two additional *Arabidopsis* lines, stably expressing aequorin targeted to the thylakoid lumen and thylakoid membrane, respectively. A new and rapid procedure to obtain autotrophic cell cultures containing chloroplasts was set up, based on the use of phytohormone (2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine)-enriched agarized medium for the germination of *A. thaliana* seeds **[Chapter 4]**.

Ca^{2+} measurement assays carried out in photosynthetic cell suspension cultures expressing aequorin targeted to the thylakoid system confirmed their suitability to monitor [Ca^{2+}] variations just outside the thylakoids and within the thylakoid lumen in response to environmental stimuli. Interestingly, in resting conditions Ca^{2+} was found to be maintained

in the thylakoid lumen at a concentration (~ 0.5 μM) which is 3÷5 fold higher than in the chloroplast stroma (0.1÷0.15 μM).

In view of the importance of the light signal for the physiology of plants, the effect of light-to-dark transition on intracellular Ca^{2+} levels was evaluated in the *A. thaliana* transgenic plant lines stably expressing the different aequorin chimeras and maintained under a 16 h light/8 h dark cycle. A comparison between the Ca^{2+} traces recorded in the different subcellular locations suggested that the thylakoid lumen might serve as a Ca^{2+}-releasable store for the Ca^{2+} fluxes observed in the stroma, as it has previously been hypothesized. Interestingly, the transient stromal Ca^{2+} signal in response to light-to-dark transition was observed only in chloroplasts, but not in non-green plastids of *A. thaliana* cell cultures, further strengthening the notion that the light off-induced Ca^{2+} fluxes are limited to chloroplasts only. Moreover, the use of nigericin, a ionophor catalyzing the exchange of H^+/K^+, indicated the likely involvement of a Ca^{2+}/H^+ antiporter, previously hypothesized to be localized at the thylakoid membranes, in the dark-induced Ca^{2+} uptake into the thylakoid lumen **[Chapter 5]**.

To investigate the involvement of putative Ca^{2+}-permeable channels localized at plastid membranes in the organellar Ca^{2+} fluxes evoked by different environmental stimuli, in collaboration with Prof. I. Szabò (Padova, Italy) an *A. thaliana* mutant line knock-out for a plastid homologue of the mammalian mitochondrial calcium uniporter (MCU) was stably transformed with the construct encoding stroma-targeted aequorin. Ca^{2+} measurement assays demonstrated the occurrence of stromal [Ca^{2+}] increases characterized by higher amplitudes in the MCU ko plant line, in comparison with the wild-type, in response to different stimuli. These unexpected results suggest that the plastidial MCU homologue might not be located at the plastid envelope, but rather at the thylakoid membrane, being involved in the dissipation of stromal Ca^{2+} signals (*via* mediation of Ca^{2+} influx into the thylakoid lumen) rather than in their generation **[Chapter 6]**.

In summary, the complex toolkit of chloroplast-targeted aequorin probes that was set up and successfully applied in this work turned out to be an effective and versatile tool to monitor plastidial Ca^{2+} dynamics in a sensitive and accurate way, providing evidence for the ability of these plant-specific organelles to evoke specific Ca^{2+} signals in response to a plethora of environmental stresses. Monitoring of Ca^{2+} dynamics in chloroplasts as well as in non-green plastids in response to different abiotic and biotic signals revealed the occurrence of stimulus-specific Ca^{2+} signals, characterized by unique kinetic parameters. In particular, evidence was provided for dark-stimulated intrachloroplast Ca^{2+} fluxes,

which suggested a new scenario for light/dark-induced Ca^{2+} transport inside these organelles.

In the last part of my PhD I carried out a six-month research period at the Marine Biological Association of the UK (Plymouth, UK), under the supervision of Dr G. Wheeler. The aim of this research stay abroad was to investigate whether aspects of the Ca^{2+} transduction mechanisms of land plants are conserved in green algae. During this stay abroad the Ca^{2+}-responsive fluorescent dye Oregon Green-BAPTA and the unresponsive dye Texas Red were used to directly image changes in intracellular Ca^{2+} in the model unicellular green alga *Chlamydomonas reinhardtii* in response to relevant environmental stimuli. Information about Ca^{2+} homeostasis and signalling events in *C. reinhardtii* is scarce: indeed, there is no information about environmental stimuli that can trigger cytosolic [Ca^{2+}] changes in this freshwater/soil alga. *Chlamydomonas* cells were challenged with the same stimuli described for *Arabidopsis*, *i.e.* mannitol, H_2O_2 and NaCl. The data obtained indicated that hyperosmotic and oxidative stress did not induce any significant $[Ca^{2+}]_{cyt}$ increase. Concerning salt stress, a single rapid $[Ca^{2+}]_{cyt}$ spike was recorded, but the percentage of cells in which this event was observed (9 out of 61) was not sufficient to further investigate its nature. Subsequently, the alga was challenged with a hypoosmotic shock, perfusing 50 μM $CaCl_2$ dissolved in deionized water, that was found to induce rapid and repetitive $[Ca^{2+}]_{cyt}$ elevations in nearly 80% of the cells. By using the extracellular Ca^{2+} chelator EGTA, it was found that external free Ca^{2+} is required for hypoosmotic-induced intracellular $[Ca^{2+}]_{cyt}$ elevations. Moreover, the involvement of mechanosensitive ion channels was demonstrated by the use of specific inhibitors (*i.e.* 10 μM ruthenium red and 5 μM GsMTx4) for this type of channels. Pre-treatments with GsMTx4 caused a significant reduction in the total number of Ca^{2+} elevations and in the magnitude of the first peak in the cells that responded. The timing and the duration over 3% of the 1st peak were not significantly affected by the inhibitor.

Taken together, these results indicate that some aspects of Ca^{2+} signalling do not appear to be conserved between the green alga *Chlamydomonas* and the higher plant *Arabidopsis*, but other elements in Ca^{2+}-mediated responses to osmotic shocks are similar. These differences may be due to the different Ca^{2+} signalling toolkits found in plants and algae and/or to the different physiology of these model organisms, both belonging to the *Viridiplantae* clade. Investigation on the Ca^{2+} handling mechanisms operating in different photosynthetic organisms of phylogenetic interest may shed new light on the evolutionary history of the Ca^{2+} signalling toolkit **[Chapter 7]**.

RIASSUNTO DELLA TESI

In questa tesi sono riportati i risultati ottenuti durante il corso triennale di dottorato di ricerca in Bioscienze e Biotecnologie, *curriculum* Biologia Evoluzionistica, svolto presso l'Università degli Studi di Padova.

La mia attività di ricerca è stata mirata a chiarire come i plastidi, un'ampia famiglia di organelli che si trova sia nelle piante che nelle diverse alghe, siano coinvolti nell'omeostasi del Ca^{2+} e nelle vie di segnalazione mediate da questo ione in risposta a stimoli ambientali nella pianta modello *Arabidopsis thaliana*.

Nelle piante il Ca^{2+} viene usato come messaggero intracellulare per trasdurre un'ampia varietà di stimoli abiotici e biotici, che inducono specifici segnali Ca^{2+} intracellulari con diverse caratteristiche spazio-temporali. Le diverse *Ca^{2+} signatures* vengono successivamente decodificate da proteine sensori del Ca^{2+} in risposte metaboliche e trascrizionali stimolo-specifiche.

Per quanto riguarda la compartimentazione interna del Ca^{2+} nelle cellule vegetali, particolare attenzione è stata volta finora al vacuolo, che contiene una concentrazione relativamente alta di Ca^{2+} libero ($[Ca^{2+}]$), e per il quale un'ampia letteratura è già disponibile a proposito di trasportatori di Ca^{2+} localizzati a livello del tonoplasto. Tuttavia, recenti evidenze suggeriscono che ulteriori compartimenti intracellulari possano svolgere importanti ruoli come depositi di Ca^{2+} in cui lo ione può essere accumulato e da cui può essere rapidamente mobilizzato, come il reticolo endoplasmatico, i mitocondri ed i cloroplasti.

Per quanto riguarda i cloroplasti, tipici organelli delle cellule vegetali, è nota l'importanza del Ca^{2+} in alcuni processi fisiologici. Tuttavia, il ruolo del Ca^{2+} nei cloroplasti e nei plastidi in generale è ancora poco chiaro. Per comprendere meglio l'integrazione dei plastidi nelle vie di segnalazione mediate dal Ca^{2+} nella cellula vegetale, è essenziale monitorare le dinamiche di Ca^{2+} all'interno del organello in modo sensibile e preciso, e identificare i meccanismi di mobilizzazione dello ione situati nelle membrane di questi organelli.

Nel corso della mia attività di dottorato sono state messe a punto colture cellulari in sospensione di *Arabidopsis thaliana* che esprimono stabilmente l'equorina, sensore bioluminescente per il Ca^{2+}, localizzato in diversi compartimenti dei cloroplasti, in particolare la membrana esterna dell'involucro plastidiale, lo stroma, la membrana tilacoidale e il lume tilacoidale. Piante di *Arabidopsis* stabilmente trasformate con i

costrutti che codificano le diverse equorine chimeriche sono state rese disponibili e messe a punto per questo progetto in collaborazione con la Prof.ssa U. Vothknecht (Monaco, Germania).

Innanzitutto, colture cellulari eterotrofe in sospensione, contenenti amiloplasti come tipo funzionale di plastidi, e che esprimono stabilmente equorina nel citoplasma, nella membrana esterna dell'involucro plastidiale e nello stroma, rispettivamente, sono state trattate con stimoli abiotici e biotici la cui trasduzione del segnale mediata dal Ca^{2+} è nota, come lo stress ossidativo, la salinità, la siccità, lo shock da freddo e gli oligogalatturonidi (frammenti pectici della parete cellulare vegetale rilasciati durante l'attacco da patogeni). Misurazioni di Ca^{2+} basate sull'equorina hanno dimostrato l'induzione di variazioni di $[Ca^{2+}]$ specifiche per ogni stimolo e caratterizzate da parametri cinetici differenti in ogni localizzazione subcellulare. Inoltre, il confronto tra le tracce Ca^{2+} ha indicato un ruolo essenziale svolto dai plastidi nella dissipazione dei diversi segnali di Ca^{2+} citosolici.

La messa a punto di colture cellulari in sospensione eterotrofe ed autotrofe che esprimono stabilmente equorina indirizzata allo stroma ha consentito una dissezione del diverso contributo di amiloplasti e cloroplasti, due diversi tipi funzionali di plastidi, nelle dinamiche di Ca^{2+} nell'organello in risposta a segnali ambientali. Sebbene le risposte Ca^{2+} di plastidi non verdi e cloroplasti adattati al buio siano risultate molto simili, differenze significative nell' ampiezza delle specifiche variazioni di Ca^{2+} plastidiali (in particolare in risposta alla salinità e siccità) sono emerse quando l'attività fotosintetica dei cloroplasti è stata riattivata dalla luce. Le peculiarità nelle risposte Ca^{2+} agli stimoli ambientali suggeriscono che i diversi tipi funzionali di plastidi potrebbero svolgere diversi ruoli in diversi organi della pianta in termini di adattamento ai cambiamenti delle condizioni ambientali, soprattutto quando questi organelli sono in un particolare stato metabolico **[Capitolo 3]**.

Le informazioni sulle dinamiche di Ca^{2+} plastidiali in risposta a stimoli ambientali sono state integrate grazie alla realizzazione di due linee supplementari di *Arabidopsis* che esprimono stabilmente l'equorina indirizzata al lume tilacoidale e alla membrana tilacoidale, rispettivamente. Una nuova metodica è stata messa a punto per ottenere colture cellulari autotrofe, contenenti cloroplasti, basata sull'uso di fitormoni (acido 2,4-diclorofenossiacetico e 6-benzilaminopurina) nel mezzo agarizzato utilizzato per la germinazione dei semi di *A. thaliana* **[Capitolo 4]**.

Misure di Ca^{2+} in risposta a stimoli ambientali in colture cellulari fotosintetiche esprimenti equorina nel sistema tilacoidale sono state effettuate per confermare l'idoneità

delle linee al monitoraggio di variazioni di [Ca^{2+}] all'esterno dei tilacoidi e all'interno del lume tilacoidale. I risultati hanno indicato che, in condizioni di riposo, nel lume tilacoidale la [Ca^{2+}] è mantenuta ad una concentrazione (~0.50 μM) di 3÷5 volte superiore a quella nello stroma dei cloroplasti (0.10÷0.15 μM).

In considerazione dell'importanza dello stimolo luminoso per la fisiologia delle piante, l'effetto della transizione luce-buio sui livelli di [Ca^{2+}] intracellulare è stato valutato in linee transgeniche di *A. thaliana* che esprimono stabilmente le diverse equorine chimeriche e mantenute ad un ciclo di 16 h luce / 8 h buio. Il confronto tra le tracce Ca^{2+} registrate nelle diverse localizzazioni subcellulari ha suggerito che il lume tilacoidale possa essere utilizzato sia come compartimento di rilascio del Ca^{2+} per i flussi osservati nello stroma, come precedentemente ipotizzato, ma che svolga anche un ruolo nello spegnimento dei flussi stromali. È inoltre interessante notare che il segnale Ca^{2+} stromale in risposta alla transizione luce-buio è stato osservato solo nei cloroplasti, ma non in plastidi di colture cellulari eterotrofe, rafforzando ulteriormente l'idea che il buio induca flussi di Ca^{2+} limitatamente ai cloroplasti. Inoltre, l'uso dell'inibitore nigericina, uno ionoforo che catalizza lo scambio di H^+/K^+ ed elimina il gradiente protonico attraverso la membrana tilacoidale, ha indicato il probabile coinvolgimento di un sistema di antiporto Ca^{2+}/H^+ nell'uptake di Ca^{2+} nel lume tilacoidale indotto dal buio **[Capitolo 5]**.

In collaborazione con la Prof.ssa I. Szabò (Padova, Italia), è stato considerato il coinvolgimento di putativi canali permeabili al Ca^{2+} localizzati a livello delle membrane plastidiali nei flussi Ca^{2+} evocati da diversi stimoli ambientali. Una linea di *A. thaliana* mutante *knock-out* per un omologo plastidiale dell'uniporto di calcio mitocondriale (MCU), di recente caratterizzato nei mammiferi, è stata trasformata per esprimere stabilmente l'equorina nello stroma dei plastidi. Misurazioni di Ca^{2+} in queste linee hanno permesso di dimostrare variazioni di [Ca^{2+}] stromale caratterizzate da maggiori ampiezze nella linea mutante, rispetto al *wild-type*, in risposta a diversi stimoli. Questi risultati inaspettati suggeriscono che l'omologo MCU plastidiale potrebbe non essere localizzato a livello dell'involucro plastidiale, come precedentemente ipotizzato, bensì nella membrana tilacoidale, essendo coinvolto nella dissipazione di segnali Ca^{2+} stromali piuttosto che nella loro generazione **[Capitolo 6]**.

Riassumendo, il complesso kit di sonde basate sull'equorina indirizzate al cloroplasto si è rivelato uno strumento efficace e versatile per monitorare le dinamiche di Ca^{2+} nell'organello in modo sensibile e preciso, fornendo evidenze per quanto riguarda la capacità dei plastidi di evocare segnali Ca^{2+} specifici in risposta ad un'ampia varietà di

stimoli ambientali. Il monitoraggio delle dinamiche di Ca^{2+} nei cloroplasti e in plastidi non verdi in risposta a differenti stimoli abiotici e biotici ha rivelato l'induzione di segnali Ca^{2+} stimolo-specifici, caratterizzati da parametri cinetici unici. In particolare, è stata fornita evidenza per flussi di Ca^{2+} intracloroplastici indotti dal buio, che suggeriscono un nuovo scenario per il trasporto di Ca^{2+} all'interno di questi organelli indotto dalla transizione luce-buio.

Durante l'ultima parte del mio dottorato ho effettuato un periodo di ricerca di sei mesi presso l'Associazione di Biologia Marina del Regno Unito (Plymouth, UK), sotto la supervisione del Dott. G. Wheeler. Lo scopo di questo soggiorno di ricerca all'estero è stato quello di verificare se alcuni aspetti della trasduzione di segnali mediata dal Ca^{2+} delle piante terrestri siano conservati nelle alghe verdi. Durante questo soggiorno all'estero il colorante fluorescente sensibile al Ca^{2+} Oregon Green-Bapta e il colorante non sensibile al Ca^{2+} Texas Red sono stati usati per misurare le variazioni della [Ca^{2+}] intracellulare in risposta a rilevanti stimoli ambientali nell'alga verde unicellulare *Chlamydomonas reinhardtii*. Sono a tutt'oggi scarse le dati riguardanti eventi di segnalazione mediati dal Ca^{2+} in *C. reinhardtii*: non ci sono infatti informazioni riguardanti stimoli ambientali che inneschino cambiamenti citosolici nella [Ca^{2+}] in questa alga d'acqua dolce. Cellule di *Chlamydomonas* sono stimolate con gli stessi stress descritti per *Arabidopsis*, cioè mannitolo, H_2O_2 e NaCl, e i dati ottenuti hanno indicato come lo stress ossidativo ed iperosmotico non abbiano indotto alcun significativo aumento nella [Ca^{2+}] citosolica. Riguardo allo stress salino, esso ha risultato indurre singole e rapide elevazioni di Ca^{2+}, ma la percentuale di cellule in cui è stato osservato questo evento (9 su 61) non è stata sufficiente per approfondirne la natura. Successivamente, l'alga è stata trattata con uno shock ipoosmotico, mediante la perfusione di 50 mM $CaCl_2$ in acqua deionizzata, che è risultato indurre rapide e ripetitive elevazioni nella [Ca^{2+}] citosolica in quasi l'80% delle cellule. Usando il chelante di Ca^{2+} extracellulare EGTA, è stato verificato che il Ca^{2+} libero esterno è necessario per gli innalzamenti intracellulari dello ione indotti dallo stress ipoosmotico. Inoltre, il coinvolgimento di canali ionici meccanosensibili è stato dimostrato mediante l'uso di inibitori specifici per questo tipo di canali (10 µM rosso rutenio e 5 µM GsMTx4). Il pretrattamento con GsMTx4 ha causato una riduzione significativa nel numero totale di innalzamenti di [Ca^{2+}] e nell'ampiezza del primo picco nelle cellule che hanno risposto, mentre la durata del primo picco e il momento in cui è stato osservato non sono stati influenzati dalla presenza dell'inibitore.

Nell’ insieme, questi risultati indicano come alcuni aspetti delle vie di segnalazione in cui il Ca^{2+} è coinvolto non sembrino essere conservati tra *Chlamydomonas* e *Arabidopsis*, ma che altri elementi delle risposte agli shock osmotici mediate dal Ca^{2+} possono essere simili. Queste differenze potrebbero essere dovute ai diversi meccanismi di omeostasi e signalling del Ca^{2+} che si trovano nelle piante e nelle alghe e/o alla diversa fisiologia di questi organismi modello, appartenenti entrambi alla gruppo delle *Viridiplantae*. Le indagini sui meccanismi di controllo della [Ca^{2+}] intracellulare in diversi organismi fotosintetici interessanti dal punto di vista filogenetico potrebbero far luce sulla storia evolutiva delle vie di segnalazione mediate dal Ca^{2+} **[Capitolo 7]**.

CHAPTER 1.

Introduction

1.1 Origin and evolution of Ca^{2+} homeostasis and Ca^{2+} signalling

In the early stages of life, primitive living cells had to face several problems to survive in an inhospitable environment. One of the most important physiological challenges was to maintain an internal milieu that could allow biochemical reactions to occur. In these terms, cells had to keep ion concentrations in their cytoplasm in a range compatible with life. After oxygen, silicon, aluminium and iron, calcium is the most abundant element on Earth's surface, where it is commonly found under the form of carbonates (CO_3^{2-}), and it is quantified in about 400 mg/l in seawater. Among all elements present in the seawater, Ca^{2+} is one of the most dangerous. In fact, in biological systems Ca^{2+} reacts with proteins and nucleic acids, affects the integrity of lipid membranes and initiates the precipitation of phosphates, forming insoluble salts. This latter effect is the most problematic for living beings, as phosphates are used for adenosine triphosphate biosynthesis, that is the molecular unit of currency in biological systems. In these conditions, the very first unicellular organisms that have evolved on our planet found themselves in a highly dangerous situation. In fact, they had to maintain an intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) at low levels (around 100 nM), compatible with metabolic processes, and so with life. In this context, cells evolved active systems to extrude the excess of Ca^{2+} from the internal space, thus allowing for the establishment of a high steep concentration gradient between intra- and extracellular environment (~10000-20000 times) (Sanders et al., 1999; Case et al., 2007). This electrochemical gradient was then exploited by cells to generate Ca^{2+} signals in response to various stimuli, generating rapid and transient elevations in the cytosolic concentration of the ion, mediated by Ca^{2+}-permeable channels located at cellular membranes (Case et al., 2007) (Fig. 1).

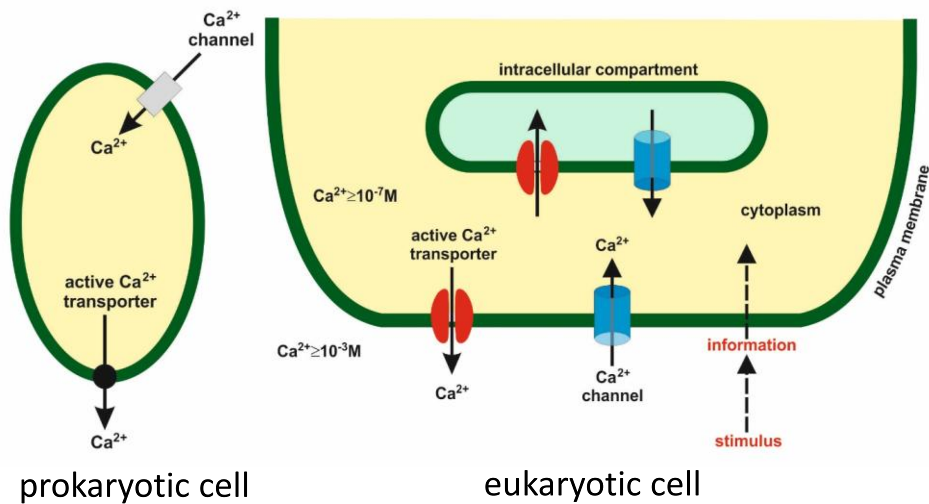


Fig. 1 Schematic representation of the Ca^{2+} homeostatic and signalling machinery in prokaryotic and eukaryotic cells. The evolution of intracellular compartmentalization added a further level of complexity and led to a more fine-tuned regulation of intracellular [Ca^{2+}] in eukaryotic cells.

With the evolution of eukaryotic cells, and so with the appearance of intracellular compartments, cells had the possibility to store the overabundance of Ca^{2+} in internal organelles. Active and passive Ca^{2+} transporters, together with the possibility to mobilize the ion from internal and external stores, allow cells to perform a fine tuned regulation of $[Ca^{2+}]_i$.

If from one side the ability of Ca^{2+} to interact with biological molecules can be problematic for cells, this characteristic has given evolution the workbench for the development of a system based on the interaction of Ca^{2+} with molecules, used to decode specific Ca^{2+} signals triggered by stimuli. As a matter of fact, organisms exploit this feature not only to complex Ca^{2+} in intracellular stores, but also to make this ion interact with proteins that act as sensors in the Ca^{2+}-mediated signal transduction. Ca^{2+} binding proteins acting as Ca^{2+} sensors are characterized by the EF-hand motif, a helix-loop-helix structure in which the amino acids composing the loop form a specific Ca^{2+}-binding domain. After the interaction with Ca^{2+}, these proteins undergo a conformational change that take to their interaction with downstream effectors, leading to a specific response for every stimulus (Dodd et al., 2010; Hashimoto & Kudla, 2011).

The universality of Ca^{2+} as an intracellular messenger is also based on its wide versatility. The Ca^{2+} signalling toolkit in cells is composed by many components that can be used in different combinations, leading to a vast variety of spatial and temporal signals (Dodd et al., 2010). This versatility is exploited to control many different physiological

processes, such as fertilization, cell proliferation and development in all eukaryotic cells (Berridge et al., 2000).

1.2 Ca^{2+} as an intracellular messenger in plants

Ca^{2+} is an intracellular transducer of primary importance in all organisms, as it is involved in many signalling pathways. In plants, Ca^{2+} signalling transduces a wide variety of abiotic and biotic stimuli that have been shown to evoke specific intracellular spatio-temporal Ca^{2+} signals, which are further transduced by Ca^{2+} sensor proteins into transcriptional and metabolic responses (Dodd et al., 2010).

Ca^{2+} signals are produced by the influx of calcium ions into the cytosol from the external environment and/or from intracellular compartments through Ca^{2+}-permeable channels. Ca^{2+} channels can be voltage-dependent, activated by either hyper- or depolarization of membranes, ligand-gated if molecules, such as some amino acids (glutamate, serine, glycine and asparagine in the case of the glutamate receptor homologues-GLRs), cyclic nucleotides (CNGC), nicotinic acid adenine dinucleotide phosphate (NAADP), cyclic adenosine diphosphate ribose (cADPR) or inositol trisphosphate ($InsP_3$), activate them, or mechanosensitive, activated by membrane stretch. They have different localizations, as they can be found on the plasma membrane or on membranes of intracellular compartments (Jammes et al., 2011). Regarding the extrusion of Ca^{2+} from the cytosol, different active Ca^{2+} transporters, *i.e.* Ca^{2+}-ATPases and Ca^{2+}/H^+ exchangers, are used by cells to eliminate the excess of Ca^{2+} and to modulate and switch-off cytosolic Ca^{2+} signals by pumping the ion in the extracellular milieu or into internal Ca^{2+} stores. Accumulation of Ca^{2+} in intracellular compartments, in addition to refill Ca^{2+}-mobilizing pools for signal transduction, may also regulate specific metabolic reactions taking place inside organelles (Berridge et al., 2000).

In plants, transient increases in intracellular [Ca^{2+}] activate signal transduction cascades that lead to specific responses to numerous biotic and abiotic stimuli (Sanders et al., 2002; Dodd et al., 2010). The information transduced by spatial and temporal variations in cytosolic [Ca^{2+}] are then decoded by Ca^{2+} binding proteins, conceptually classified in sensor relays and sensor responders. The first group includes calmodulins (CaMs) and CaM-like proteins (CMLs) that, upon Ca^{2+} binding, undergo extensive conformational changes, thereby altering downstream target activities via Ca^{2+}-dependent protein-protein interaction. The latter set of proteins is composed by enzymes (Ca^{2+}-dependent protein kinases-CDPKs, and the bimolecular complexes formed by calcineurin

B-like proteins-CBLs- and CBL interacting protein kinases-CIPKs) whose activity is regulated by the binding with Ca^{2+}. Sensor responders translate the information encoded in the Ca^{2+} signals into phosphorylation of specific target proteins (Hashimoto & Kudla, 2011) (Fig. 2).

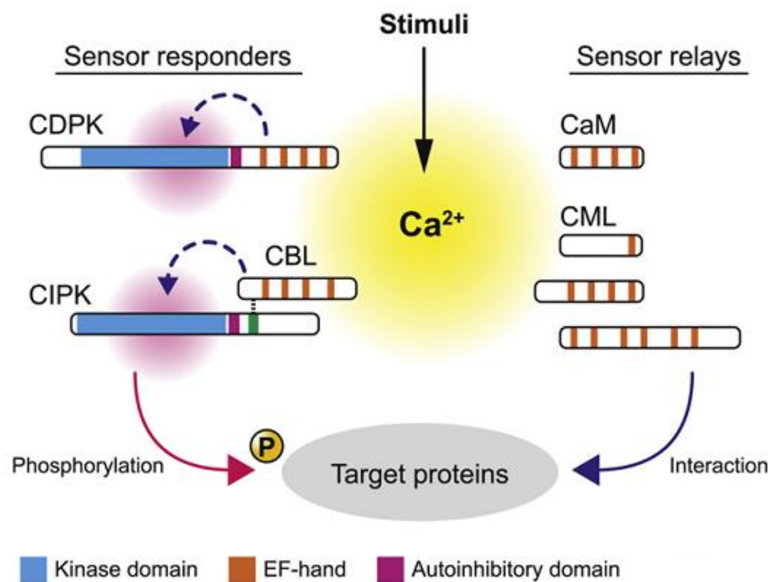


Fig. 2 Schematic overview of the mechanisms by which Ca^{2+}sensor proteins decode Ca^{2+} signals. Sensors relays (CaM and CMLs), which have no enzymatic functions, regulate target proteins via Ca^{2+}-dependent protein-protein interactions. By contrast, sensor responders (CDPKs and CBL-CIPK complexes) possess Ca^{2+}-regulated protein kinase activity (from Hashimoto & Kudla, 2011).

All these Ca^{2+} sensor proteins differently act downstream on target proteins, such as enzymes, membrane transporters and transcription factors, that then lead to changes in metabolic activity and gene expression, so as to carry out appropriate physiological responses for the different stimuli (Dodd et al., 2010) (Fig. 3). Every stimulus, whose signal transduction is known to be mediated by Ca^{2+}, evoke a typical Ca^{2+} signature, whose duration, frequency, amplitude of transient $[Ca^{2+}]_i$ increases and spatial localization allow a specific stimulus-response coupling (McAinsh & Pittman, 2009).

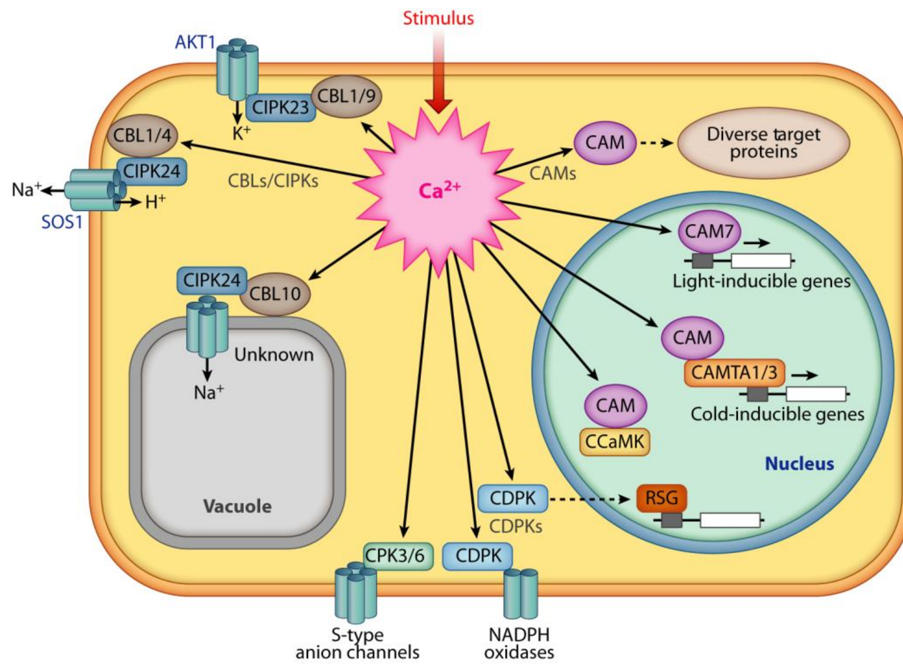


Fig. 3 Examples of different interactions between Ca^{2+}-binding proteins acting as Ca^{2+} sensors and their partners. Ca^{2+} sensors interact with target proteins in different membranes and/or subcellular compartments. The high number of interactors is one of the elements that contribute to provide stimulus-specific responses (from Dodd et al., 2010).

1.3 Intracellular compartments of the plant cell involved in Ca^{2+} homeostasis and Ca^{2+}-based signal transduction

In the recent years, research about Ca^{2+}-mediated signalling in plants has been mainly focused on the generation and decoding of cytosolic Ca^{2+} signals, but little information is available about the ability of the different intracellular compartments to act as Ca^{2+}-mobilizable sites. In these terms, particular attention has been payed so far mainly to the vacuole, that fills great part of the intracellular space in mature cells and contains a high free [Ca^{2+}]. On the other hand, increasing evidence suggests that other intracellular compartments, such as the endoplasmic reticulum (ER), the apoplast, the nucleus, peroxisomes, mitochondria and chloroplasts (Fig. 4), may tightly control their internal [Ca^{2+}] and contribute to intracellular Ca^{2+} handling, thereby regulating metabolic processes and signalling events in the cytoplasm and in the entire cell (Stael et al., 2012; Nomura & Shiina, 2014) (Fig. 5).

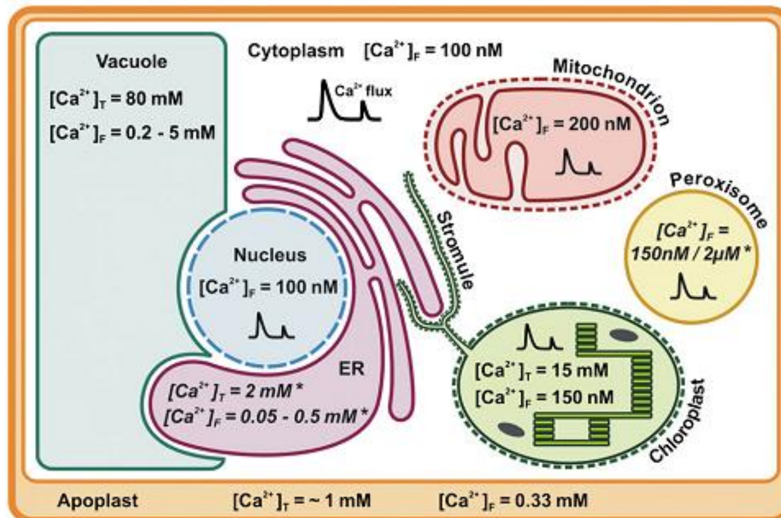


Fig. 4 Intracellular compartments known to be involved in Ca^{2+} homeostasis and Ca^{2+} signalling in the plant cell. Reported $[Ca^{2+}]$ values have been observed in many studies, as well as Ca^{2+} fluxes in response to biotic and abiotic stimuli, illustrated by a double peak-shaped symbol. $[Ca^{2+}]_T$ and $[Ca^{2+}]_F$ refer to total and free resting $[Ca^{2+}]$, respectively. $[Ca^{2+}]$ values in the ER and in peroxisomes are taken from the animal field and are marked with an asterisk (*) (from Stael et al., 2012).

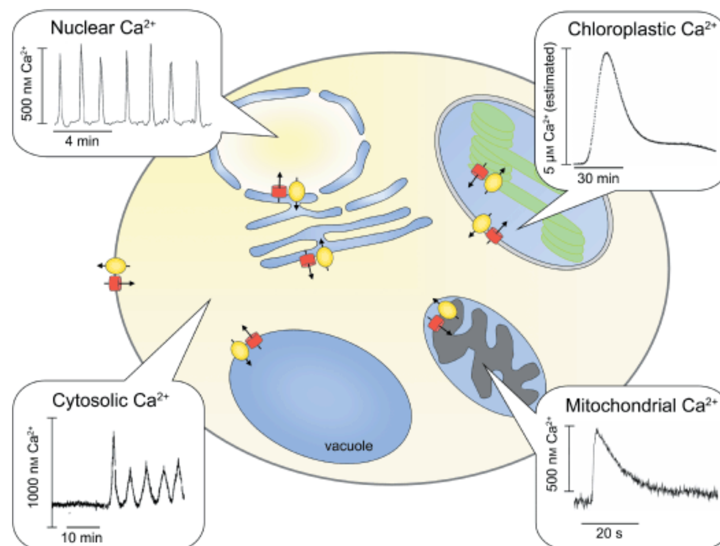


Fig. 5 Specificity of Ca^{2+} signals in different subcellular localizations induced by various abiotic and biotic stimuli (Nod-factor in the nucleus; light-to-dark transition in chloroplasts; external Ca^{2+} in the cytosol; touch in mitochondria). The activity of both Ca^{2+}-permeable channels (red rectangles) and active Ca^{2+} transporters (yellow ovals) underlie the generation of stimulus-specific Ca^{2+} signatures by generating and switching-off Ca^{2+} fluxes (from McAinsh & Pittman, 2009).

1.3.1 Vacuole

This acidic compartment fills more than the 80% of the intracellular space in mature plant cells and it is considered to be the main Ca^{2+} storage compartment, as it contains 0.2-5 mM free Ca^{2+}, where total Ca^{2+} can reach 80 mM. Great part of it is sequestered by organic acids, such as malate, citrate and isocitrate, and it is not readily available for Ca^{2+}-mediated signalling events. Clear evidence indicates that vacuolar Ca^{2+} can take part in

signal transduction pathways influencing, for example, the activity of ionic transporters localized on the tonoplast (Pottosin & Schönknecht, 2007). Active Ca^{2+} transporters have been identified at the vacuolar membrane, namely Ca^{2+}-ATPases and Ca^{2+}/H^{+} exchangers. Moreover, the ligand-gated channels, activated by cADPR and $InsP_3$, respectively, and two voltage-gated channels, one activated by hyperpolarization and one activated by depolarization (the slow vacuolar channel) have been characterized by biochemical and electrophysiological studies (Peiter, 2011). This latter one, encoded by the *Two Pore Channel 1* (*TPC1*) gene, is the only vacuolar Ca^{2+}-permeable channel cloned to date (Peiter et al., 2005). Recent evidence indicates that the vacuolar ion channel TPC1 is involved in salt stress-induced Ca^{2+} waves associated with rapid, long-distance root-to-shoot signalling in *Arabidopsis thaliana* (Choi et al., 2014).

1.3.2 Endoplasmic reticulum

Not much information is available so far regarding the involvement of the endoplasmic reticulum (ER) in Ca^{2+} homeostasis and Ca^{2+}-mediated signalling in plants. Total $[Ca^{2+}]$ in the ER lumen in animal cells is estimated to be about 2 mM, whereas free Ca^{2+} is 50-500 µM. On the other hand, evidence about the actual $[Ca^{2+}]$ in the ER lumen in plant cells is still missing. No homologue genes coding for $InsP_3$ receptors and ryanodine receptors are present in higher plants genomes (Wheeler & Brownlee, 2008; Verret et al., 2010; Edel & Kudla, 2015). However, biochemical studies carried out by measuring $^{45}Ca^{2+}$ fluxes in ER-enriched microsomal vesicles provided evidence for the presence of Ca^{2+}-mobilizing mechanisms activated by NAADP (Navazio et al., 2000) and cADPR (Navazio et al., 2001) at ER membranes. Furthermore, an important role in Ca^{2+} homeostasis in the ER has been demonstrated to be played by calreticulin, the main ER Ca^{2+}-buffering protein in both animal and plant cells (Mariani et al., 2003). It has recently been suggested that the ER may be involved in Ca^{2+} fluxes activated by some stimuli, such as the salt stress (Bonza et al., 2013). The role of ER in accumulating and releasing Ca^{2+} is of great interest in the plant field, as this compartment physically interacts with stromules, dynamic stroma-filled tubules extending from the plastid envelope (Schattat et al., 2011), suggesting the possibility of bidirectional Ca^{2+} fluxes between these two compartments (Fig. 6).

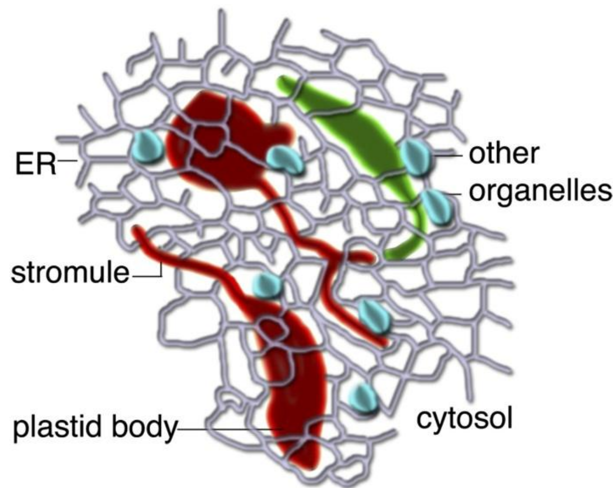


Fig. 6 Schematic representation of the physical interaction between stromules and ER (from Mathur et al., 2013).

1.3.3 Apoplast

The apoplast, which is formed by the continuum of cell walls of adjacent plant cells, can be considered as a *bona fide* Ca^{2+}-storage compartment in plant cells, since it accumulates variable [Ca^{2+}] (10 µM-10 mM), but great part of it is complexed with negatively charged carboxyl groups of pectins. This relatively high [Ca^{2+}] has to be tightly regulated, as the structure and rigidity of the cell wall is controlled by Ca^{2+}-mediated cross-linking of pectins, involved, for example, in guard cells movement for the opening/closure of stomata. Different types of Ca^{2+} channels are involved in mediating Ca^{2+} release from the apoplast in the cytosol, and they include voltage-dependent as well as ligand-gated (*i.e.* CNGCs and GLRs) Ca^{2+} channels located at the plasma membrane (Stael et al., 2012).

1.3.4 Nucleus

It has been demonstrated that nuclear Ca^{2+} signals triggered in response to different environmental stimuli may affect gene expression (Mazars et al., 2011). Ca^{2+} can influence transcription levels through interactions with Ca^{2+}-binding transcription factors, calmodulin-binding transcription activators (CAMTAs) (Whalley et al., 2011) or Ca^{2+}-dependent protein kinases (CDPKs). The nuclear resting free [Ca^{2+}] is estimated to be about 100 nM, a value very close to the concentration recorded in the cytosol. Interestingly, numerous stimuli have been demonstrated to trigger cytosol-independent Ca^{2+} fluxes in the nucleus, suggesting that the nuclear envelope may be involved in the generation of the observed Ca^{2+} changes (Pauly et al., 2000). Two cationic channels have been identified at the nuclear envelope, called Castor and Pollux, whose involvement in

Ca^{2+}-signalling has been inferred since as amino acid mutations closed to the selectivity filter of these transporters impaired perinuclear and intranuclear Ca^{2+} spiking during legume root endosymbioses (Charpentier et al., 2008).

1.3.5 Peroxisomes

Information about Ca^{2+} fluxes in peroxisomes is still relatively scarce, both in the animal and in the plant field. Two resting free [Ca^{2+}] have been recently reported in animal peroxixomes, namely 150 nM and 2 μM (Stael et al., 2012). Plant peroxisomes have been demonstrated to undergo Ca^{2+} fluxes themselves, and the intra-peroxisomal Ca^{2+} rises were found to enhance the detoxification of H_2O_2 *in vivo* through the stimulation of catalase activity (Costa et al., 2010). These data unveil the possibility for an active role of Ca^{2+} in peroxisome own metabolic activities, *i.e.* the Ca^{2+}-dependent scavenging system.

1.3.6 Mitochondria

The involvement of mitochondria in intracellular Ca^{2+} handling is well studied in animal cells, in which moderate increases in [Ca^{2+}] have been shown to positively influence ATP production by regulating the activity of the major limiting enzymes of the citric acid cycle (McCormack et al., 1990), whereas overaccumulation of Ca^{2+} in mitochondria leads to the induction of the apoptotic process. On the other hand, very little information is so far available regarding the role of mitochondria in Ca^{2+} homeostasis and signalling in plants. Free [Ca^{2+}] in plant mitochondria is estimated to be about 200 mM (Logan & Knight, 2003), and various environmental stimuli, such as cold shock, hyperosmotic, mechanical stimulation and oxidative stress, were demonstrated to trigger [Ca^{2+}] variations in the mitochondrial matrix, characterized by kinetic parameters that were different from those observed in the cytosol, suggesting that mitochondrial and cytosolic Ca^{2+} dynamics are differentially regulated (Logan & Knight, 2003; Loro et al., 2012). These differences suggest that mitochondria are not just passive Ca^{2+} sinks for the dissipation of cytosolic signals, but that they are able to control and fine-tune their own internal [Ca^{2+}] (Stael et al., 2012).

Information about Ca^{2+} channels at plant mitochondrial membranes is still scarce, but the *Arabidopsis* genome contains six genes with a low homology to the human mitochondrial calcium uniporter (MCU) (De Stefani et al., 2011); of these potential MCU isoforms, five have a predicted mitochondrial localization, whereas one has a potential chloroplast localization (Stael et al., 2012).

1.3.7 Chloroplasts

It has long been known that chloroplasts are able to accumulate large quantities of Ca^{2+} and that Ca^{2+} is involved in the modulation of chloroplast metabolism. The total [Ca^{2+}] in chloroplasts has been estimated to be 15 mM or higher. Nevertheless, since a high [Ca^{2+}] is known to affect the activity of some Calvin-Benson cycle enzymes, and to cause phosphate precipitation, most of the chloroplast Ca^{2+} has to be bound to proteins in the stroma and in the thylakoid system: indeed, free [Ca^{2+}] in the chloroplast stroma is estimated to be about 150 nM (Johnson et al., 1995).

Concerning the photosynthetic process, some CO_2 fixation enzymes, such as fructose-1,6-bisphophatase and sedoheptulose bisphosphatase, are activated by low [Ca^{2+}] and effectively inhibited by elevated [Ca^{2+}] (Rocha & Vothknecht, 2012). This demonstrates that chloroplasts have a precise request for Ca^{2+}, but it is essential that [Ca^{2+}] in this organelle is finely tuned. Ca^{2+} is also known to regulate the photosynthetic light reactions, as Ca^{2+} is an essential cofactor of the oxygen-evolving complex and binds the 8 kDa subunit of the ATP synthase, thereby controlling the photosynthetic proton flow and ATP production (Zakharov et al., 1993; Ifuku et al., 2010). Even additional plastidial physiological processes, not directly related to photosynthesis, are affected by Ca^{2+}. For example, the import of nuclear-encoded proteins was shown to be regulated by CaM/Ca^{2+} via the TOC and TIC (translocons at the outer/inner envelope of chloroplasts) complex (Chigri et al., 2005 and 2006).

Concerning the involvement of chloroplasts in Ca^{2+}-mediated signal transduction in response to environmental stimuli, Ca^{2+} fluxes have been detected in the organellar stroma upon light-to-dark transition. This transient stromal Ca^{2+} rise is proposed to play some role in the inhibition of photosynthetic CO_2 fixation during the night. The origin of the observed plastidial Ca^{2+} fluxes remain to be established (Sai & Johnson, 2002).

More recently, Ca^{2+} fluxes in the chloroplast stroma have been demonstrated to occur also in response to pathogen-associated molecular patterns (PAMPs). In particular, flg22, a peptide derived from bacterial flagellin, triggered a transient [Ca^{2+}] change in the cytosol first, and then in the chloroplast stroma. This stromal Ca^{2+} rise was found to be required for the induction of the expression of defence-related nuclear genes, suggesting the presence of a complex intracellular signalling system mediated by Ca^{2+} that might activate defence responses in plants (Nomura et al., 2012). Evidence was provided for the involvement in the PAMP-induced stromal Ca^{2+} transients and defence gene expression of

a thylakoid membrane-localized protein, named calcium-sensing receptor (CAS), which is able to bind Ca^{2+} with low affinity and high capacity (Nomura et al., 2008 and 2012). Even the phosphorylation of the photosynthetic complexes by the thylakoid membrane protein kinases STN7 and STN8 may contribute to the Ca^{2+} buffering ability of the organelle (Nomura & Shiina, 2014). The participation of chloroplasts, and plastids in general, to the plant Ca^{2+} signalling network would also require the presence of organellar Ca^{2+} sensors, able to decode plastidial Ca^{2+} signals. Research studies focused on the identification of potential CaMs, CaML proteins, Ca^{2+}-regulated kinases and their targets are currently ongoing (Stael et al., 2012). A schematic overview of the Ca^{2+}-regulated network in chloroplasts is represented in Fig. 7.

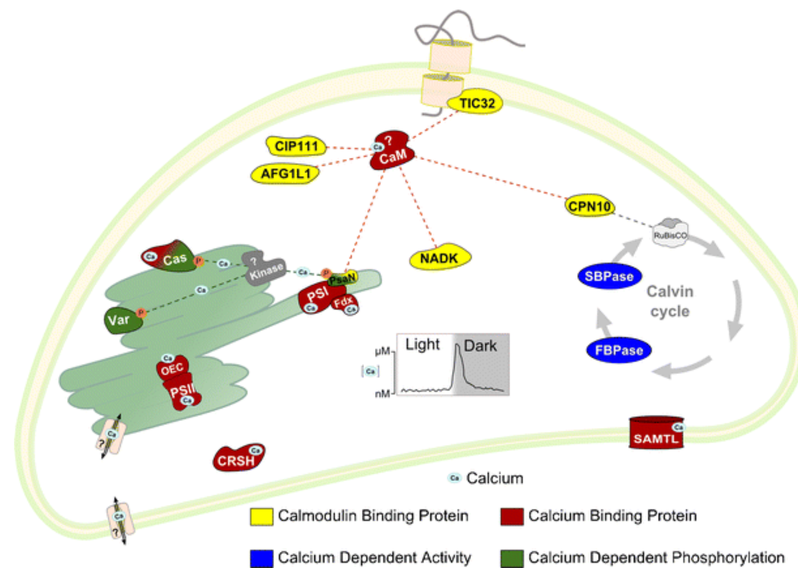


Fig. 7 Schematic representation of the chloroplast Ca^{2+}-mediated regulation network. In particular, proteins and processes involved in the organellar Ca^{2+} signalling are reported (from Rocha & Vothknecht, 2012).

Concerning Ca^{2+} transporters localized at chloroplast membranes, information is still scarce. Biochemical evidence has indicated a Ca^{2+}/H^{+} antiporter activity at the thylakoid membrane, that could be involved in the uptake of Ca^{2+} into the thylakoid lumen via the thylakoid proton gradient (Ettinger et al., 1999). Recent studies have shown the presence of a ligand-gated Ca^{2+} channel AtGLR3.4, homologue to glutamate receptors in animals, in the plastid envelope (Teardo et al., 2010 and 2011). An electrophysiological approach demonstrated that glutamate and glutamine induce a current inhibited by non-selective cation channel blockers, such as La^{3+}, 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), that are considered specific inhibitors for glutamate receptors. More recently, another member of the plant glutamate receptor family (AtGLR3.5) has been demonstrated to have a dual localization in mitochondria and

chloroplasts (Teardo et al., 2015). It has been hypothesised that these Ca^{2+}-permeable channels might contribute to generate Ca^{2+} signals in chloroplasts.

Of particular interest is also a putative plastidial Ca^{2+}-permeable channel homologue to the mammalian mitochondrial Ca^{2+} uniporter (MCU) (De Stefani et al., 2011). As reported before, the *Arabidopsis* genome contains six genes encoding 6 plastidial MCU isoforms, one of which (Atg66650) is predicted to be targeted to chloroplasts (Stael et al., 2012). Although the 6 putative MCU isoforms share a rather low homology to the human MCU, the pore region with two transmembrane helices connected by a DIME (human) or DVME (*Arabidopsis*) motif is highly conserved (Fig. 8).

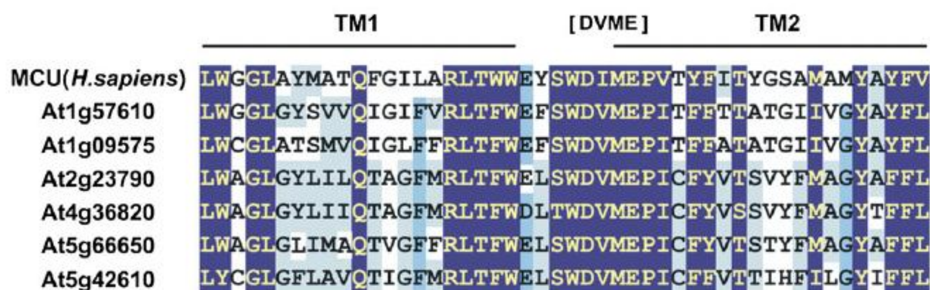
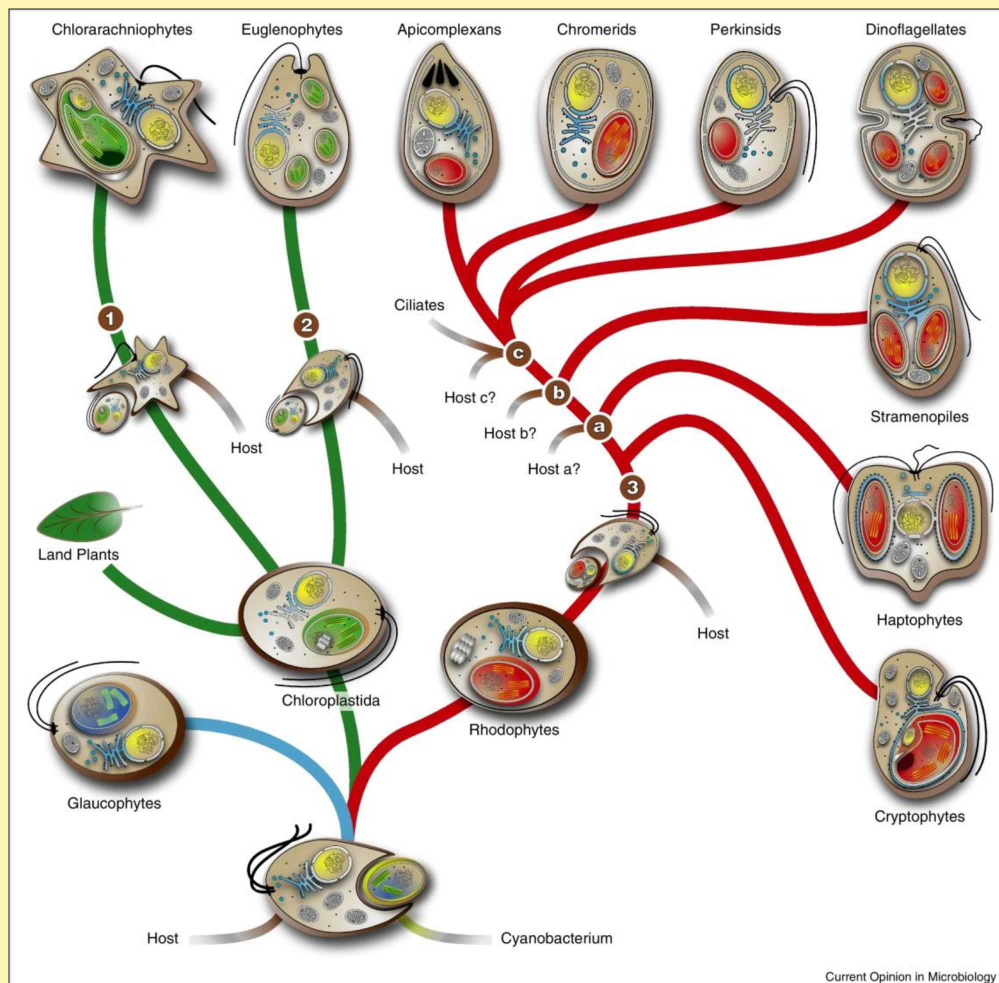


Fig. 8 Amino acidic sequence alignment for the human MCU pore-forming domain with the 6 *Arabidopsis* isoforms. The highly conserved regions in the transmembrane helices (TM1 and TM2) connected by a loop domain are highlighted. (from Stael et al., 2012).

Evolutionary origin of plastids

The theory for the endosymbiotic origin of plastids, a diverse family of organelles which are found ubiquitously in land plants and various algae, dates back over 100 years, and it assumes that they were once free-living prokaryotes and became organelles of eukaryotic cells about 1.2 billion years ago. It is hypothesized that photosynthetic bacteria were phagocytised by a heterotrophic eukaryotic host that did not completely digest its prey, but semi-autonomous organelles evolved inside their host. Phylogenetic analyses have demonstrated that modern cyanobacteria are the prokaryotes most closely-related to the ancient photosynthetic ancestors of plastids, but these analyses also confirmed that there was a gene flux form the ex-free-living organism to the genome of the host. In fact, plastids tend to retain a miniaturized prokaryotic chromosome encoding about 200 proteins, despite these organelles harbour approximately 2000 proteins. This discrepancy is generally explained by endosymbiotic gene transfer, that involved gene transfer to the nucleus. Transferred genes would have acquired sequences for the their targeting back to the plastid, and a protein import machinery would have also been formed (Jarvis & López-Juez, 2013). Genes that are still present in the genome of the plastids are believed to be essential, as they encode for proteins involved in the electron transport chain of the bioenergetic organelle or for the ribosomes required for their synthesis (Zimorski et al., 2014).

Plastids in current photosynthetic organisms present a differential number of membranes surrounding the organelle, indicating the occurrence of more than one endosymbiotic event. Primary plastids, that have a double membrane originated from the inner and outer membrane of the prokaryote, are found in green plants, green algae containing chlorophylls *a* and *b* as their photosynthetic pigments, and red algae containing chlorophyll *a* and phicobiliprotein. Secondary and tertiary plastids are surrounded by more than two membrane. They represent the additional acquisition of a photosynthetic eukaryote by a non-photosynthetic eukaryotic host followed by varying levels of simplification including reduction in number of membranes bounding the secondary plastid and a loss of the nucleus of the photosynthetic eukaryote. Tertiary plastids are generated by the loss of secondary plastids from a photosynthetic eukaryote and their replacement by plastids form another source (Howe et al., 2008). The first endosymbiotic event would have given origin to Glaucophytes, Chloroplastida (the ancestor of the green lineage), and Rhodophytes. Phylogenetic reconstruction of plastid evolution is reported in the figure below.



Plastid evolution. The initial uptake of a cyanobacterium-related organism by a heterotrophic host would have led to three lineages: Glaucophytes, Chloroplastida and Rhodophytes. Chloroplastida then evolved in three green photosynthetic lineages, where higher plants and microalgae are included (from Zimorski et al., 2014).

Much evidence supports the endosymbiotic origin for plastids. The most important comes from organellar genomes and their conservation between cyanobacteria and

plastids, referring for example to similarity in operon-based gene organization, plastome-localization of the gene encoding for the small subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcS*) and the conservation of ribosomal proteins in chloroplasts. Moreover, other conserved features in plastids are: their division by binary fission, the presence of two photosystems co-working in the photosynthetic process and the evolution of photosynthetic oxygen, and, in some primitive plastids, a peptidoglycan wall (Martin et al., 2002).

1.4 Monitoring intracellular Ca^{2+} concentration and its changes during signal transduction events

Intracellular [Ca^{2+}] and its variations during signal transduction can be monitored using Ca^{2+} probes, *i.e.* molecules that can form selective complexes with Ca^{2+} ions and whose physicochemical characteristics of the free and bound forms are sufficiently different to enable their relative concentrations to be measured (Rudolf et al., 2003). The most commonly used Ca^{2+} indicators are fluorescent dyes and protein-based Ca^{2+} indicators.

Fluorescent dyes are derivatives of the Ca^{2+} chelator EGTA: the conformational change caused by Ca^{2+} binding to the carboxyl groups is transmitted to the chromophore and results in changes in the excitation/emission properties of the dye. In particular, with ratiometric dyes, such as fura-2 and indo-1, the excitation/emission spectrum changes according to free [Ca^{2+}]. On the other hand, with non-ratiometric dyes, such as fluo-4, rhod-2 and Oregon Green BAPTA, [Ca^{2+}] is determined solely by a relative increase in the fluorescent intensity (Rudolf et al., 2003). This category of Ca^{2+} reporters allow to monitor [Ca^{2+}] dynamics in single cells and to give a clear spatial information, but they cannot be targeted to subcellular compartments. Thus, only cytosolic [Ca^{2+}] and its changes can be monitored. Moreover, there can be problems associated with the loading of these dyes in plants cells, due to the presence of thick cell walls. Despite these intrinsic limitations, these dyes have been extensively used in a whole variety of Ca^{2+} signalling studies, especially in microalgae (Brownlee & Wood, 1986; Brownlee et al., 1999; Goddard et al., 2000; Wheeler et al., 2008).

The second category of Ca^{2+} probes encompasses genetically encoded Ca^{2+} indicators (GECIs), that allow to monitor [Ca^{2+}] variations even in specific subcompartments of the cells. These indicators have, in their sequence, three or four EF-hand Ca^{2+} binding sites. It is possible to specifically localize them within the cells by including defined targeting signals in the amino acidic sequence. GECIs include fluorescent (*i.e.* cameleon, camgaroo, Pericams) and luminescent (*i.e.* aequorin) Ca^{2+} sensitive probes of protein nature. The

former type is based on GFP variants fused to CaM. When CaM binds Ca^{2+}, a conformational change takes the GFP-based moieties to a closer distance: this process leads to an increase of fluorescence resonance energy transfer (FRET) between the fluorescent proteins, that is directly related to the variation in $[Ca^{2+}]$ (Fig. 9). The advantage of these Ca^{2+} reporters is the possibility to monitor Ca^{2+} in single cells and to accurately image Ca^{2+} spiking events, but they cannot be used in photosensitive compartments, as high-light intensity is needed for the excitation of probes.

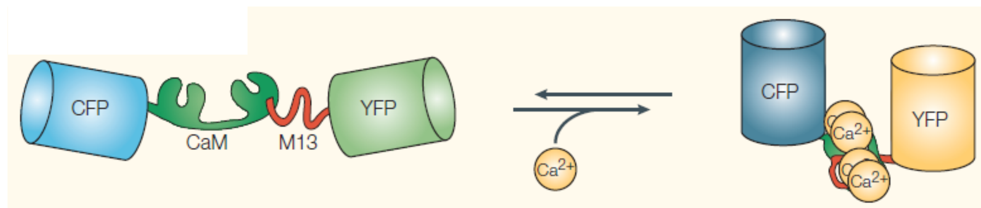


Fig. 9 Schematic model for the GFP-based Ca^{2+} indicator Cameleon (from Rudolf et al., 2003).

Aequorin, a bioluminescent Ca^{2+} reporter, is a photoprotein that requires its prostetic group coelenterazine to function as a Ca^{2+} indicator. Upon Ca^{2+} binding, aequorin undergoes a conformational change that leads to the oxidation of coelenterazine to coelenteramide and the concurrent emission of a photon of blue light (Fig. 10). The light signal is collected and converted into $[Ca^{2+}]$ values by using a reliable calibration procedure (Brini et al., 1995). The recombinant aequorin method offers several advantages, such as: a) extremely sensitive and reliable $[Ca^{2+}]$ measurements in a large dynamic range of Ca^{2+} (from 10^{-7} to 10^{-3} M); b) a high signal-to-noise ratio; c) a nearly insensitiveness to changes in pH. On the other hand this technique is not suitable for single-cell imaging studies, because of the relatively low amount of emitted light (Brini, 2008). For this reason, most aequorin-based experiments are carried out in cell populations or, in the case of plant cell signalling studies, even in entire organisms (seedlings *in toto*).

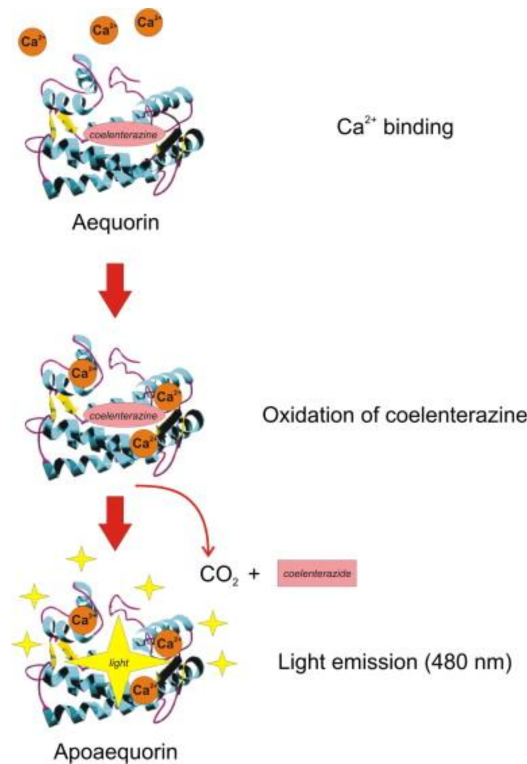


Fig. 10 Schematic representation of the mechanism that leads to luminescence emission by reconstituted aequorin (from Brini, 2008).

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CHAPTER 2.
Aim of the work

The aim of this work is to analyse the role of plastids in the plant Ca^{2+}-mediated signal transduction activated in response to different environmental stimuli, considering in particular the involvement of specific plastid sub-compartments. Current knowledge regarding chloroplasts and Ca^{2+} suggests that these organelles may be potential intracellular Ca^{2+} stores, wherein $[Ca^{2+}]$ is finely controlled because it regulates the photosynthetic process, both in the light phase and in the Calvin-Benson cycle, and because it is also involved in processes not related to photosynthesis. Nevertheless, the role of Ca^{2+} as a regulator of numerous processes that take place in the chloroplast still remains unclear. Relevant information would arise from accurate measurements of $[Ca^{2+}]$ in the different plastid sub-compartments, and of its possible changes in the organelle in response to different stimuli. Indications about the role of plastids as Ca^{2+} source and/or sink in the generation and/or dissipation of cytosolic Ca^{2+} signals, thus contributing to the modulation of Ca^{2+} signatures, are still scarce. Investigations of these organellar $[Ca^{2+}]$ changes are of high interest because intracellular Ca^{2+} dynamics are fundamental to encode for stimulus-response specificity.

Based on these considerations, the aim of this work is to monitor $[Ca^{2+}]$ and its changes in different sub-localizations of chloroplasts. Moreover, the possibility that non-green plastids and chloroplasts may display differential Ca^{2+} responses to environmental cues is also considered.

In this research study the method of recombinant aequorin, a bioluminescent Ca^{2+} reporter widely used to measure $[Ca^{2+}]$ in an extremely reliable and sensitive way in entire seedlings and cell populations, was applied to monitor chloroplast $[Ca^{2+}]$ variations. In particular, Ca^{2+} measurements were performed in the cytosolic domain close to the external membrane of the plastid envelope, in the plastid stroma, at the thylakoid membrane exposed to the stroma and in the thylakoid lumen. The targeting of the Ca^{2+} probe to the different chloroplast sub-localizations was obtained by the fusion of the aequorin cDNA with the coding sequence for protein domains specifically localized in the sub-compartments under investigation.

In Chapter 3, the involvement of two different functional types of plastids, namely chloroplasts and amyloplasts, in plant intracellular Ca^{2+} dynamics in response to environmental stimuli was analyzed. Autotrophic and heterotrophic cell suspension cultures, containing chloroplasts and amyloplasts, respectively, stably expressing aequorin in the cytosol, outer membrane of plastid envelope and in the stroma of plastids, were set up from independent lines of the model plant *Arabidopsis thaliana* stably transformed with the corresponding constructs. The obtained cell cultures were used to monitor $[Ca^{2+}]$ variations in response to NaCl, mannitol, H_2O_2, cold shock and oligogalacturonides, in the two functional types of plastids.

In Chapter 4 a new rapid method to obtain photosynthetic cell suspension cultures from *Arabidopsis* is described. This novel technique, which is less laborious and more straightforward than traditional methods, was successfully applied to set up one of the experimental systems used in the following Chapter to analyse the correct localization and functionality as Ca^{2+} probes of the newly designed aequorin chimeras targeted to the thylakoid system.

In Chapter 5 a new toolset of aequorin chimeras targeted to the thylakoid lumen and thylakoid membrane was set up in order to investigate the involvement of the thylakoid system in the Ca^{2+} homeostasis and signalling network in *Arabidopsis*. This new toolkit was assessed to be suitable to monitor $[Ca^{2+}]$ and its changes in the two chloroplast sub-compartments in response to different environmental stimuli. In particular, the transition from light to dark was found to induce a peculiar $[Ca^{2+}]$ variation in the thylakoid lumen, that led us to hypothesize a role for the thylakoid system in both the generation and dissipation of the corresponding stromal Ca^{2+} signal.

Chapter 6 deals with an analysis of the potential participation of a plant homologue of the mammalian mitochondrial Ca^{2+} uniporter (MCU), putatively localized in chloroplasts, in mediating organellar Ca^{2+} fluxes triggered in response to environmental stimuli. An *Arabidopsis* knock-out mutant defective in this Ca^{2+}-permeable channel was transformed with the construct encoding aequorin targeted to the plastid stroma, and variations in $[Ca^{2+}]$ upon challenge with relevant environmental stimuli were monitored in this knock-out line and compared with the wild-type line.

In view of the endosymbiotic origin of plastids, it would be interesting from an evolutionary point of view to investigate if the involvement of the chloroplast in the Ca^{2+}-mediated perception of environmental stimuli is conserved between different organisms that belong to the green lineage. In Chapter 7, the ability of *Chlamydomonas reinhardtii*, a

unicellular freshwater/soil green alga, to evoke intracellular Ca^{2+} changes in response to different environmental stimuli was considered. The long-term project would have been the comparison of chloroplast Ca^{2+} dynamics triggered by similar environmental stimuli in the higher plant *Arabidopsis thaliana* and in *Chlamydomonas reinhardtii*. Neverthless, whereas cytosolic Ca^{2+} changes in response to external stresses are well established in *Arabidopsis*, there is no evidence in the literature about environmental stimuli that trigger cytosolic [Ca^{2+}] variations in *Chlamydomonas*. As a consequence, the first step in such types of investigations, before considering the involvement of *Chlamydomonas* chloroplast in Ca^{2+} dynamics, had necessarily to deal with the identification of environmental cues evoking cytosolic [Ca^{2+}] elevations in the microalga. I carried out this work at the Marine Biological Association of the UK (Plymouth, UK) under the supervision of Dr G. Wheeler, during a six-month research period (February-August 2015). *Chlamydomonas* cells, loaded with the Ca^{2+}-responsive fluorescent dye Oregon Green-BAPTA and the unresponsive dye Texas Red as a reference, were challenged with stimuli previously used in this work on *Arabidopsis*, *i.e.* NaCl, mannitol and H_2O_2. As *Chlamydomonas* lives in ponds, puddles and in the soil, it possibly has to cope with dilution of its environment in heavy rainy days: thus, the alga was also challenged with a hypoosmotic shock and the nature of the observed dynamics was investigated.

CHAPTER 3.

Dissecting stimulus-specific Ca^{2+} signals in amyloplasts and chloroplasts of *Arabidopsis thaliana* cell suspension cultures

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Running Title: Dissection of plastidial calcium signals in *Arabidopsis*

Highlight: *Arabidopsis* suspension-cultured cells stably expressing aequorin in the plastid stroma were used as a suitable tool to dissect differential Ca^{2+} responses of non-green plastids *versus* chloroplasts to several environmental cues.

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Abstract

Calcium is used by plants as an intracellular messenger to decipher a plethora of environmental stimuli and respond to stresses with a fine-tuned regulation. The interest in unveiling the role of different subcellular compartments in Ca^{2+} homeostasis and signalling has been growing in the recent years. In this work, to evaluate the potential participation of non-green plastids and chloroplasts in the plant Ca^{2+} signalling network, heterotrophic and autotrophic cell suspension cultures were set up from *Arabidopsis thaliana* plant lines stably expressing the bioluminescent Ca^{2+} reporter aequorin targeted to the plastid stroma. Our results indicate that both amyloplasts and chloroplasts are involved in the generation of transient Ca^{2+} increases in the plastid stroma induced by several environmental stimuli, suggesting that these two functional types of plastids are endowed with similar mechanisms for Ca^{2+} handling. The comparison of the Ca^{2+} trace kinetics recorded in parallel in the plastid stroma, the surface of the outer membrane of the plastid envelope and the cytosol indicated an essential role played by plastids in switching off different cytosolic Ca^{2+} signals. Interestingly, a transient stromal Ca^{2+} signal in response to light-to-dark transition was observed in chloroplasts, but not amyloplasts. Moreover, significant differences in the amplitude of specific plastidial Ca^{2+} changes emerged when the photosynthetic metabolism of chloroplasts was reactivated by light. In summary, our work highlights differences between non-green plastids and chloroplasts in terms of Ca^{2+} dynamics in response to environmental stimuli.

Key words: aequorin, *Arabidopsis*, amyloplasts, calcium signals, cell cultures, chloroplasts.

Introduction

In plants Ca^{2+} is used as an intracellular messenger to transduce a plethora of abiotic and biotic stimuli. Indeed, a wide array of environmental stimuli have been shown to evoke specific intracellular spatio-temporal Ca^{2+} signals, which are further transduced by Ca^{2+} sensor proteins into transcriptional and metabolic responses (Dodd *et al.*, 2010; Whalley and Knight, 2013). Regarding the intracellular compartmentalization of the ion, research studies have been focused so far mainly on the vacuole, which is considered as the major Ca^{2+} storage compartment in the plant cell, and for which extensive knowledge is already available about Ca^{2+} transporters localized at the tonoplast (Peiter, 2011; Martinoia *et al.*, 2012; Xu *et al.*, 2015). On the other hand, the role of other intracellular compartments, such as chloroplasts, peculiar organelles of the plant cell, in orchestrating plant intracellular Ca^{2+} dynamics is still poorly understood. It has long been known that Ca^{2+} is involved in the modulation of photosynthesis (for recent review see *e.g.* Hochmal *et al.*, 2015), as well as in other plastidial processes, such as organelle division and the import of nuclear-encoded proteins (Kovacs-Bogdan *et al.*, 2010; Rocha and Vothknecht, 2012; Stael *et al.*, 2012). Nevertheless, the role of Ca^{2+} in chloroplasts, and in plastids in general, is still elusive, with only little information so far available on the involvement of plastids in Ca^{2+} homeostasis and the generation of specific Ca^{2+} signals inside the plastids (Nomura and Shiina, 2014). Moreover, it has to be considered that different functional types of plastids are present in plants, and their structural and physiological differences may lead to a differential involvement in the Ca^{2+} signalling network.

In an *in toto* experimental system, where root cells, stem cells and leaf cells concurrently operate after signal perception to orchestrate an appropriate stimulus-specific response, non-green plastids and chloroplasts could act in distinct ways in the Ca^{2+}-mediated transduction of signals. In order to better understand the involvement of different types of plastids in the Ca^{2+} signalling pathways of the plant cell, it is essential to monitor Ca^{2+} dynamics inside the organelle in a sensitive and accurate way, and to be able to safely discern the specific Ca^{2+} responses belonging to the different functional types of plastids (Jarvis and López-Juez, 2013). To allow for a dissection of Ca^{2+} responses in non-green *versus* photosynthetic plastids, in this work we have generated, from *Arabidopsis thaliana* seedlings stably expressing stroma-targeted aequorin (Mehlmer *et al.*, 2012), cell suspension cultures containing either amyloplasts or chloroplasts. Indeed, some stimulus-specific intracellular Ca^{2+} signals, sometimes limited to a specific tissue or cell type, may

be amplified in homogeneous, rapidly proliferating cell populations (Moscatiello *et al.*, 2013). The obtained data show that different environmental cues triggered [Ca^{2+}] elevations characterized by stimulus-specific kinetic parameters in the plastid stroma of both amyloplasts and chloroplasts, whereas the light-to-dark transition evoked a transient [Ca^{2+}] change in the stroma of chloroplasts only. Moreover, significant differences in the amplitude of stromal [Ca^{2+}] changes in response to specific abiotic stimuli were detected when dark-adapted chloroplasts were re-activated by light, suggesting that plastidial Ca^{2+} responses are correlated to the photosynthetic status of the organelle.

Materials and methods

Chemicals

All chemicals, if not otherwise specified, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant material

Arabidopsis thaliana ecotype Columbia (Col-0) plant lines stably expressing the Ca^{2+}-sensitive photoprotein aequorin fused to YFP (YA), and targeted to different intracellular locations were used in this study. These transgenic plant lines have been genetically transformed with plasmids encoding YA chimera targeted to: a) the plastid stroma, by fusion with the first 85 amino acids of NAPH-dependent thioredoxin reductase C at the N-terminus of YA; b) the surface of the plastid outer envelope, by the fusion of the full-length outer envelope protein 7 (OEP7) at the N-terminus of YA; c) the cytosol (excluding the nucleus), by the presence of the nuclear export signal from the heat-stable protein kinase inhibitor introduced between the cytosolic $CPK17_{G2A}$ and YA. The whole expression cassettes of the different constructs were under the control of the CaMV 35S promoter. All YA fusion vectors carried kanamycin resistance (Mehlmer *et al.*, 2012).

Set up of *A. thaliana* heterotrophic cell suspension cultures

Transgenic seeds were surface sterilized for 60 s in a 70% ethanol, 0.05% Triton-X 100 solution, 60 s in 100% ethanol and let to dry on an autoclaved Whatman paper disc for at least 10 min. Seeds were subsequently plated on half-strenght Murashige and Skoog (MS) medium, containing 1.5% sucrose, 0.8% plant agar (Duchefa Biochemie, Haarlem,

The Netherlands) and 50 µg/ml kanamycin. Cotyledons and hypocotyls from 7-day old seedlings were transferred in axenic conditions onto Callus Induction Medium (CIM) (Gamborg B5 basal medium, 0.5 g/l MES, 2% sucrose, 0.8% agar, pH 5.7, 0.5 mg/l 2,4-dichlorophenoxiacetic acid (2,4-D), 0.05 mg/l kinetin, supplemented with 50 µg/ml kanamycin, as described by Moscatiello *et al.* (2013). Callus fractions were transferred onto fresh CIM medium every 4 weeks for two to three times, and then subcultured on MS medium containing 3% sucrose, 0.8% agar, pH 5.5, 0.5 µg/ml 2,4-D, 0.25 µg/ml 6-benzylaminopurine (6-BaP) and 50 µg/ml kanamycin. Initiation of cell suspension cultures was carried out by transferring portions of well-developed calli into liquid MS medium containing the same concentrations of sucrose and plant hormones as the solid medium, supplemented with 10 µg/ml kanamycin, and gently ground to obtain an adequate fragmentation of the calli. Cell suspension cultures were maintained at 24°C with a 16/8 h light/dark photoperiod, an illumination of 25 µmol photons $m^{-2}s^{-1}$ on a rotary shaker at 80 rpm and subcultured every week.

Set up of *A. thaliana* autotrophic cell suspension cultures

Autotrophic *A. thaliana* cell suspension cultures stably expressing stromal aequorin were initiated from heterotrophic cell suspension cultures by gradually decreasing sucrose concentration from 3% to 0.5% (w/w). Moreover, they were exposed to a relatively high illumination rate (110 µmol photons $m^{-2}s^{-1}$) under an unvaried 16/8 h light/dark cycle, to further stimulate the photosynthetic activity (Hampp *et al.*, 2012).

Microscopy observations

A. thaliana seedlings and calli were observed under a Leica MZ16 F fluorescence stereomicroscope. Images were acquired with a Leica DFC 480 digital camera, using the Leica Application Suite (LAS) software. The intracellular localization of the YFP-aequorin chimeras in *A. thaliana* heterotrophic and autotrophic cell suspension cultures was analyzed using a Leica TCS SP5 II confocal laser scanning system mounted on a Leica DMI6000 inverted microscope. Excitation with the Ar laser was carried out at 488 nm and the emitted fluorescence was detected at 505-530 nm for YFP and at 680-720 nm for chlorophyll. Cells were also observed under a Leica DM5000 B microscope after staining with Lugol solution for starch granules detection. Images were acquired with a Leica DFC425 C digital camera, using the LAS software.

Pulse Amplitude Modulation (PAM) analyses

Photosynthetic activity of autotrophic cell cultures was analyzed in 6-day-old cultures, by placing samples in a Closed FluorCam 800 MF (Photon Systems Instruments, Drasov, Czech Republic). PAM fluorimetric analysis provides the photosynthetic efficiency expressed as F_v/F_m value, where F_v is the difference between the maximal (F_m) and the basal (F_0) fluorescence of chlorophyll. Chlorophyll fluorescence images were recorded by a CCD camera, data analysis was carried out by using the FluorCam 7 software (Photon Systems Instruments).

Aequorin-based Ca^{2+} measurement assays

Mid-exponential phase (4-day-old) *A. thaliana* cell suspension cultures were reconstituted overnight with 5 µM coelenterazine (Prolume Ldt, Pinetop, Arizona, USA) on a shaker at 80 rpm, 24°C in the dark. After extensive washing, a 50 µl-cell aliquot was then used at the luminometer (Electron Tubes Limited, Middlesex, UK) to monitor intracellular Ca^{2+} changes in response to selected environmental stimuli. Intracellular [Ca^{2+}] dynamics were monitored after the treatment of cell samples with 10 mM hydrogen peroxide (oxidative stress), 0.3 M NaCl (salt stress), 0.6 M mannitol (drought), 3 volumes of ice-cold cell culture medium (cold shock), and 20 µg/ml oligogalacturonides (OGs) with a degree of polymerization from 10 to 15, pectic fragments of the plant cell wall that mimic a pathogen attack (Moscatiello *et al.*, 2006). All stimuli, except cold shock, were prepared as 2-fold concentrated solutions in MS medium containing the appropriate concentration of sucrose for heterotrophic and autotrophic cell cultures, respectively. Moreover, the effect of light-to-dark transition on stromal [Ca^{2+}] in amyloplasts and chloroplasts was tested, by transferring suspension-cultured cells in the luminometer chamber at the end of the 16 h light cycle and measuring dark-induced [Ca^{2+}] changes. Ca^{2+} responses to the previously mentioned stimuli were also monitored after 16 h light reactivation of the photosynthetic metabolism of chloroplasts. At the end of each experiment all the remaining aequorin pool was discharged by injecting into the cell samples 0.33 M $CaCl_2$, 10% ethanol. The light signal was collected and converted off-line into Ca^{2+} concentration values using a computer algorithm based on the Ca^{2+} response curve of aequorin (Brini *et al.*, 1995).

Statistical analysis

Data are shown as means ± S.E. of at least three independent experiments, and the differences between groups were assessed by Student's *t*-test.

Results

Ca^{2+} measurement assays in *Arabidopsis thaliana* heterotrophic cell cultures revealed the participation of amyloplasts in Ca^{2+} handling and in the generation of stromal Ca^{2+} signals

Plant cell cultures are widely used as a useful and versatile experimental system that allows for the dissection of the complexity of the plant organism *in toto*, permitting the analysis of a wide array of physiological processes at the cellular level. In particular, suspension-cultured cells have been shown to be a valuable tool to analyze many different Ca^{2+}-based signal transduction pathways (Navazio *et al.*, 2007a, b; Lachaud *et al.*, 2010; Amelot *et al.*, 2011; Manzoor *et al.*, 2012). In this work, *in vitro* cultures were obtained after the dedifferentiation of cotyledons and hypocotyls from *Arabidopsis thaliana* lines that stably express the bioluminescent Ca^{2+} reporter aequorin in the plastid stroma, the cytosolic surface of the outer membrane of the plastid envelope, and the cytosol, respectively. All constructs used for plant transformation carried a YFP-aequorin fusion (Mehlmer *et al.*, 2012), that enabled an easier screening of the most promising seedlings to generate *in vitro* cultures, based on YFP fluorescence (Fig. 1A). About one month after the transfer of axenic explants onto dedifferentiating medium (CIM), fluorescent calli were formed (Fig. 1B); they were subsequently transferred into liquid medium (MS medium containing 3% sucrose, supplemented with 0.5 µg/ml 2,4-D and 0.25 µg/ml 6-BaP), to get cell suspension cultures (Supplementary Fig. S1). Lugol staining, which highlighted evident starch granules inside plastids (Fig. 1C), together with the lack of chlorophyll fluorescence (Fig. 2, middle column), indicated that the cell suspension cultures contained amyloplasts as functional type of plastids. Confocal microscopy analyses confirmed the correct intracellular localizations of all the YFP-aequorin chimeras, targeted to the plastid stroma (Fig. 2A), the outer membrane of the plastid envelope (Fig. 2B), and the cytosol (Fig. 2C), respectively.

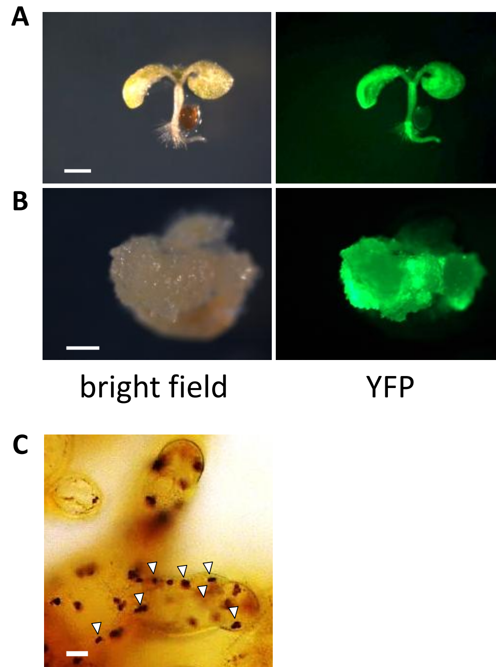
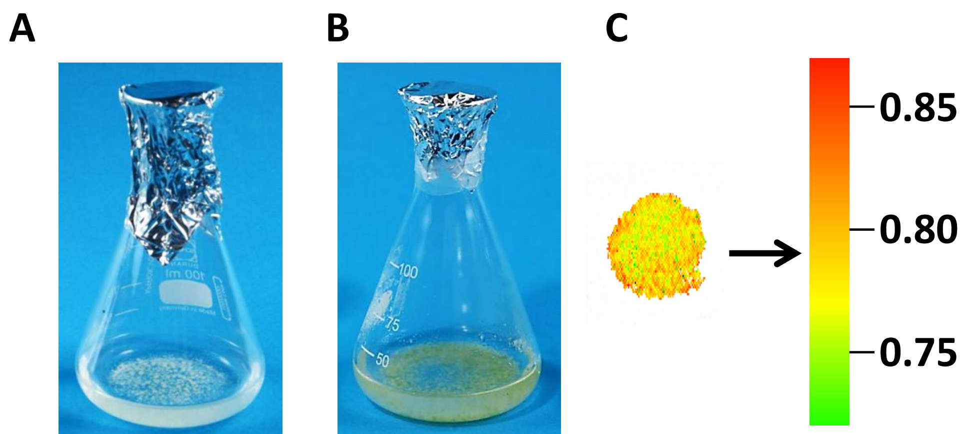


Fig. 1. Set up of *Arabidopsis thaliana* heterotrophic cell cultures stably expressing the Ca^{2+}-sensitive photoprotein aequorin in the plastid stroma. Explants from seedlings expressing the YFP-aequorin chimera and showing a good level of fluorescence (A) were placed on Callus Induction Medium to make hypocotyls and cotyledons dedifferentiate. After about one month explants produced well-developed calli (B) that were subsequently transferred on solid MS medium and then in liquid MS medium containing 3% sucrose and the appropriate concentrations of phytohormones, to obtain rapidly proliferating cell suspension cultures. The same method was applied to set up heterotrophic cell cultures stably expressing aequorin in the plastid outer envelope and cytosol. A-B: Bar, 1 mm. (C) Staining of suspension-cultured cells with Lugol solution. Starch granules inside amyloplasts are indicated by white arrowheads. Bar, 10 µm.



Supplementary Fig. S1. *A. thaliana* heterotrophic (A) and autotrophic (B) cell suspension cultures stably expressing aequorin in the stroma. C) PAM imaging analysis of *A. thaliana* autotrophic cell suspension cultures (F_v/F_m value = 0.79).

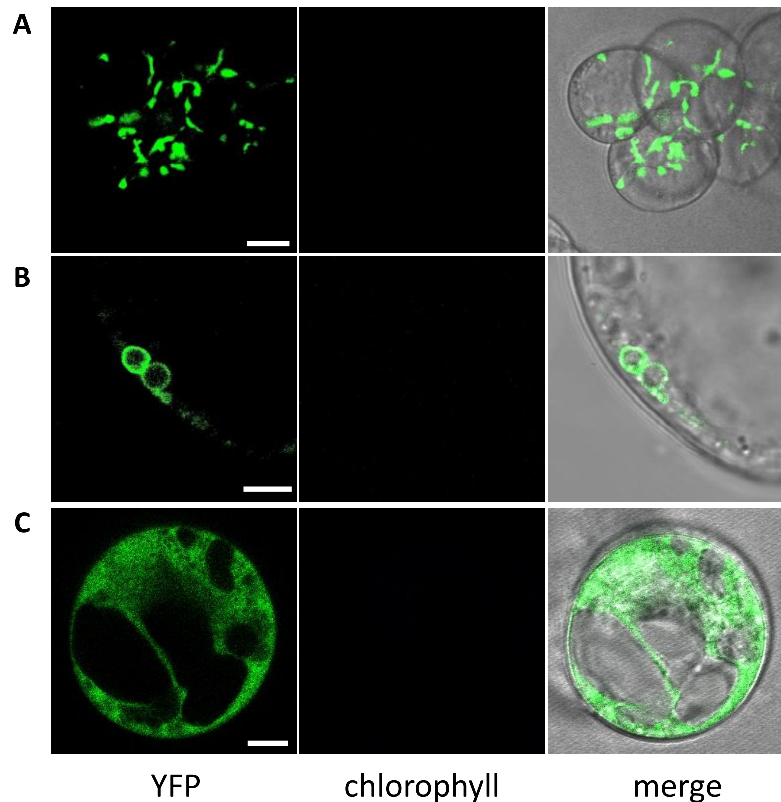


Fig. 2. Confocal microscopy analysis of YFP-aequorin localization in *A. thaliana* suspension-cultured cells stably expressing the different chimeras in the plastid stroma (A), outer envelope (B) and cytosol (C). Bars: 10 µm (A, C), 5 µm (B).

Heterotrophic cell cultures were challenged with different environmental stimuli whose Ca^{2+}-mediated signal transduction is well established, in order to monitor in parallel Ca^{2+} dynamics in the bulk cytosol, the cytosolic microdomain just outside plastids (by means of the aequorin chimera targeted to the cytosolic surface of the plastid outer envelope) and the organelle stroma. The concentration of Ca^{2+} in all these subcellular locations was found to be between 100 and 200 nM in resting conditions, indicating a similarly low Ca^{2+} level in both cytosol and plastid stroma (Fig. 3A). Upon treatment with different abiotic and biotic stimuli, transient increases in [Ca^{2+}] were recorded in all subcellular localizations, characterized by different kinetic parameters (Fig. 3B-F) as illustrated by the shown representative experiments. Since all chemicals were dissolved in cell culture medium, cells were challenged with an equal volume of isoosmotic cell culture medium as a touch control (mechanical perturbation), which induced a rapid and small increase of [Ca^{2+}] that was quickly dissipated (Fig. 3A, insert).

In response to oxidative stress (10 mM H_2O_2, Fig. 3B) a cytosolic [Ca^{2+}] increase was recorded peaking at 0.24 ± 0.06 µM after 49 ± 5 s (n=5), whereas in the cytosolic microdomain close to the surface of amyloplasts (outer envelope) the maximum [Ca^{2+}]

value observed was recorded at 0.35 ± 0.02 μM after 54 ± 4 s (n=3). In the amyloplast stroma the oxidative stress induced a slower [Ca^{2+}] rise that did not reach a plateau but continued to increase in the considered time interval. Interestingly, the ascending stromal Ca^{2+} trace well matched with the decrease in cytosolic [Ca^{2+}] level, suggesting a potential involvement of plastids in the dissipation of the Ca^{2+} transient in the cytosol.

Cell treatment with salt stress (0.3 M NaCl, Fig. 3C) or drought stress (0.6 M mannitol, Fig. 3D) induced fast [Ca^{2+}] increases in all the considered subcompartments. In response to both stimuli transient microdomains of high [Ca^{2+}] were recorded close to the organelle outer envelope, characterized by a higher amplitude (1.26 ± 0.16 μM, n=5, with 0.3 M NaCl and 1.37 ± 0.21 μM, n=6, with 0.6 M mannitol) than the corresponding [Ca^{2+}] changes in the bulk cytosol (0.66 ± 0.11 μM, n=5, with 0.3 M NaCl and 0.92 ± 0.11 μM, n=8, with 0.6 M mannitol). Interestingly, the decrease of [Ca^{2+}] level in both the bulk cytosol and cytosolic microdomain at the plastid surface temporally coincided with the increase of [Ca^{2+}] in the stroma (peaking at 0.79 ± 0.08 μM, n=6, for 0.3 M NaCl and 0.92 ± 0.11 μM, n=8, for 0.6 M mannitol), suggesting again a role for non-green plastids as Ca^{2+} sinks involved in the modulation of intracellular Ca^{2+} signals.

Cold shock, simulated by injection of 3 volumes of ice-cold cell culture medium, determined an immediate [Ca^{2+}] elevation in all the three subcellular localizations, characterized by different magnitudes (Fig. 3E). The highest increase (3.45 ± 0.19 μM at 6 ± 1 s, n=4) was recorded in the cytosol, whereas the Ca^{2+} peaks monitored at the plastid surface (1.61 ± 0.29 μM, n=5) and stroma (0.79 ± 0.13 μM, n=4) were found to be progressively reduced, although characterized by an equally fast kinetics. Indeed it is known that cold shock activates a rapid [Ca^{2+}] increase in the cytosol due to the influx of Ca^{2+} from the extracellular medium and a release of the ion from the vacuole (Knight *et al.*, 1996).

Oligogalacturonides (OGs) with a degree of polymerization from 10 to 15, *i.e.* pectic fragments of the plant cell wall that mimic a pathogen attack (Moscatiello *et al.*, 2006), were used as a biotic stimulus (Fig. 3F). OGs (20 μg/ml) induced a remarkable [Ca^{2+}] increase in the cytosol that peaked at 1.67 ± 0.08 μM after 27 ± 5 s (n=3) and lasted for about 5 min. A similar response was observed at the outer membrane surface subdomain, albeit with a little lower amplitude (1.00 ± 0.19 μM, n=4). OGs were even found to evoke a transient Ca^{2+} increase in the plastid stroma, characterized by a similar kinetic but lower amplitude (0.39 ± 0.04 μM, n=7). Taken together, the data obtained indicate that in resting conditions [Ca^{2+}] is kept at similar values in the cytosol and in the stroma of amyloplasts in

A. thaliana suspension-cultured cells, and that various environmental stresses evoke differential stromal [Ca^{2+}] signals, specific for each stimulus. Moreover, analysis of the kinetics of the stromal Ca^{2+} transients demonstrated that, at least in some case (oxidative stress, salinity, drought), plastids seem to be involved in the dissipation of the stimulus-induced cytosolic Ca^{2+} increases, rather than in the generation of the intracellular Ca^{2+} rises.

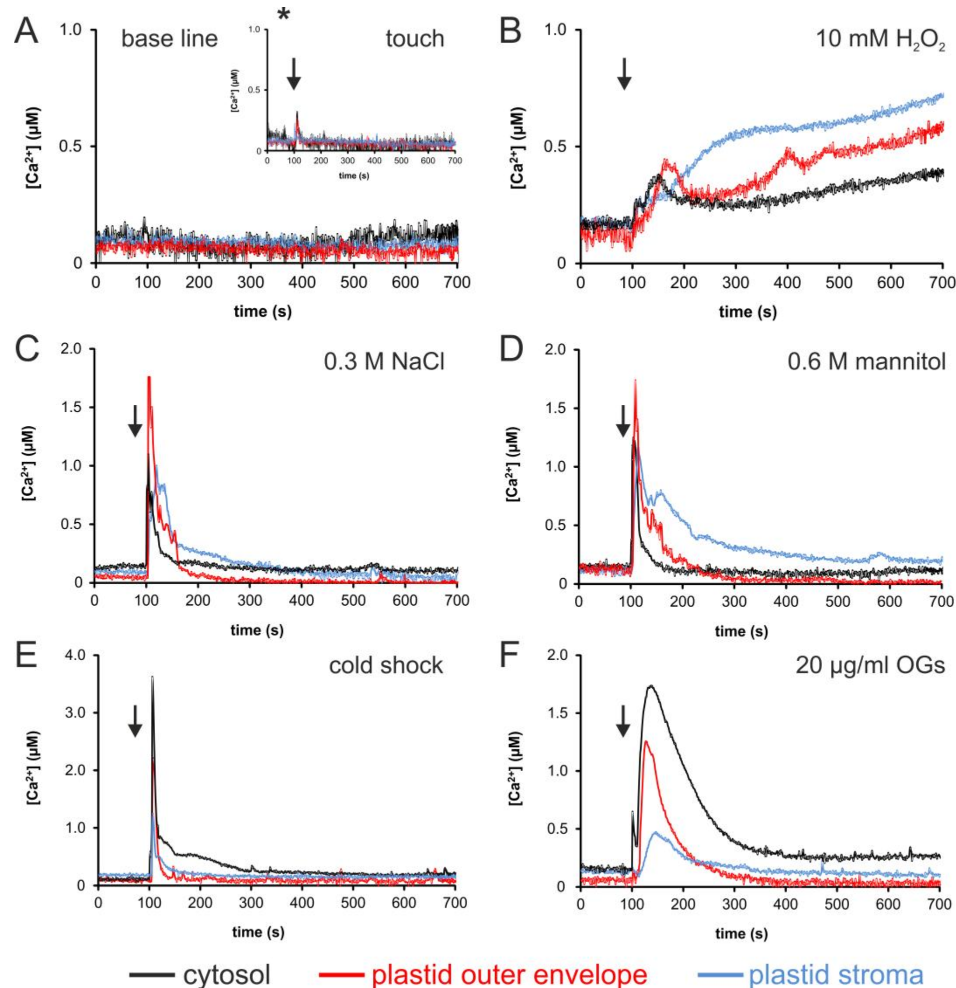


Fig. 3. Monitoring of intracellular Ca^{2+} dynamics in response to different environmental stimuli in *A. thaliana* heterotrophic cell suspension cultures containing amyloplasts and stably expressing aequorin in the cytosol (black trace), plastid outer envelope (red trace) and stroma (blue trace). Ca^{2+} measurement assays were carried out in resting conditions (A) and in response to abiotic (B-E) and biotic (F) stimuli, as indicated in each panel of the figure. In the insert (asterisk) the effect of the injection of an equal volume of cell culture medium on [Ca^{2+}] is reported as a control for stimulus administration. Cells were challenged with the different stimuli after 100 s (arrow). These and the following Ca^{2+} traces are representative of at least three independent experiments giving similar results.

Differential Ca^{2+} signals in chloroplasts *versus* amyloplasts are recorded when the photosynthetic metabolism of autotrophic cell cultures is reactivated by light

In order to compare stromal Ca^{2+} dynamics between amyloplasts and chloroplasts, *Arabidopsis* photoautotrophic cell cultures expressing aequorin in the plastid stroma were set up (Supplementary Fig. S1). To this aim, the light intensity to which heterotrophic cell

suspension cultures were exposed under a 16 h light / 8 h dark photoperiod was increased from 25 to 110 µmol photons $m^{-2}s^{-1}$ and the supply of organic carbon in the plant cell culture medium was gradually decreased from 3% to 0.5% (w/w) sucrose, to stimulate the photosynthetic activity (Hampp *et al.*, 2012).

Confocal microscopy analysis showed that in the autotrophic cell line, unlike the corresponding heterotrophic one, plastids displayed a remarkable level of chlorophyll autofluorescence, confirming the presence of chloroplasts as functional type of plastids (Fig. 4B). It should be noted that stromules, dynamic stroma-filled tubules continuously extending and retracting from plastids, were much more evident in amyloplasts than chloroplasts from *Arabidopsis* suspension-cultured cells (Fig. 4A and Supplementary Videos S1 and S2). PAM assays demonstrated the high photosynthetic efficiency of the autotrophic cell cultures, expressed as F_v/F_m value (0.75 ± 0.03, n=3), where F_v is the variable fluorescence given by the difference between maximal and basal fluorescence of chlorophyll, and F_m is the maximal fluorescence (Supplementary Fig. S1). In the heterotrophic cell cultures the calculation of F_v/F_m parameter is not possible, because they contain amyloplasts that do not possess thylakoids, and therefore lack chlorophyll and photosynthetic activity.

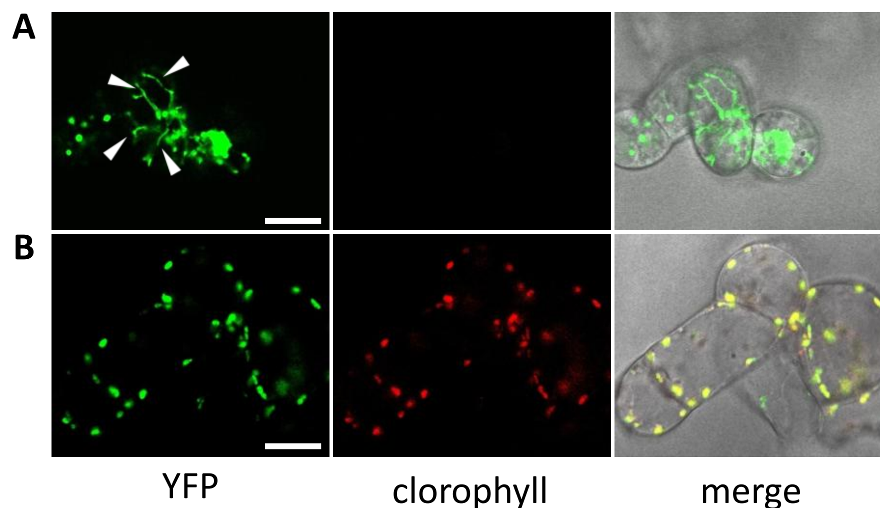
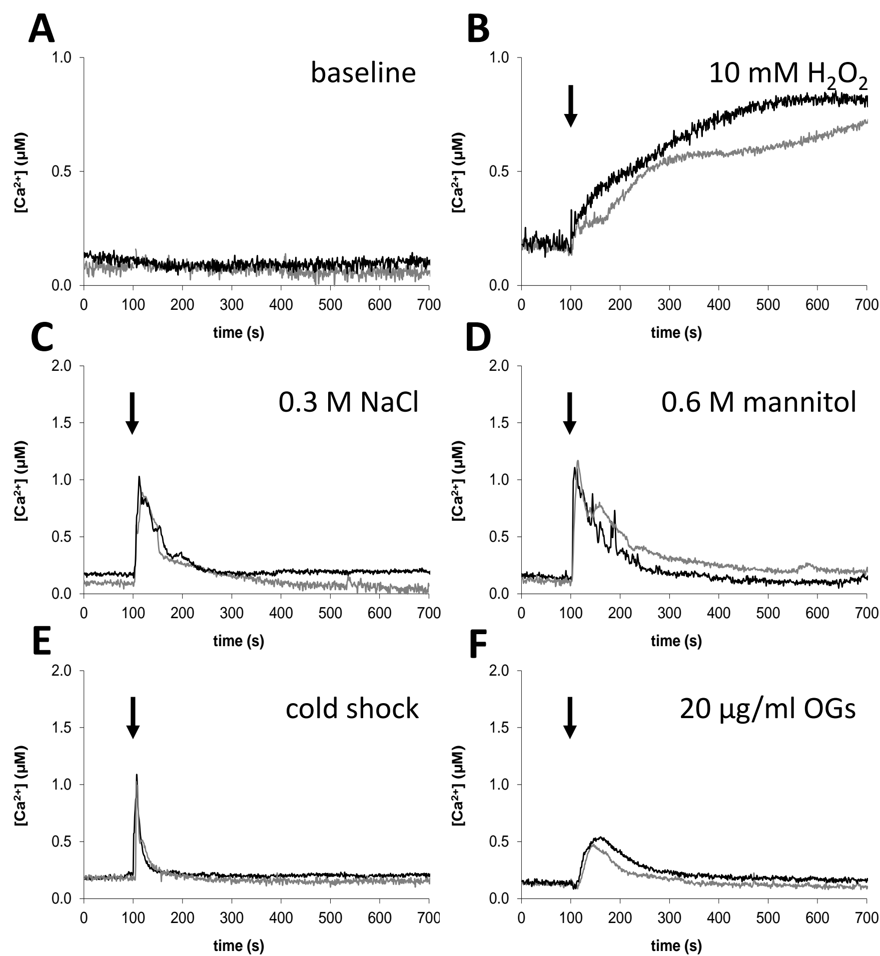


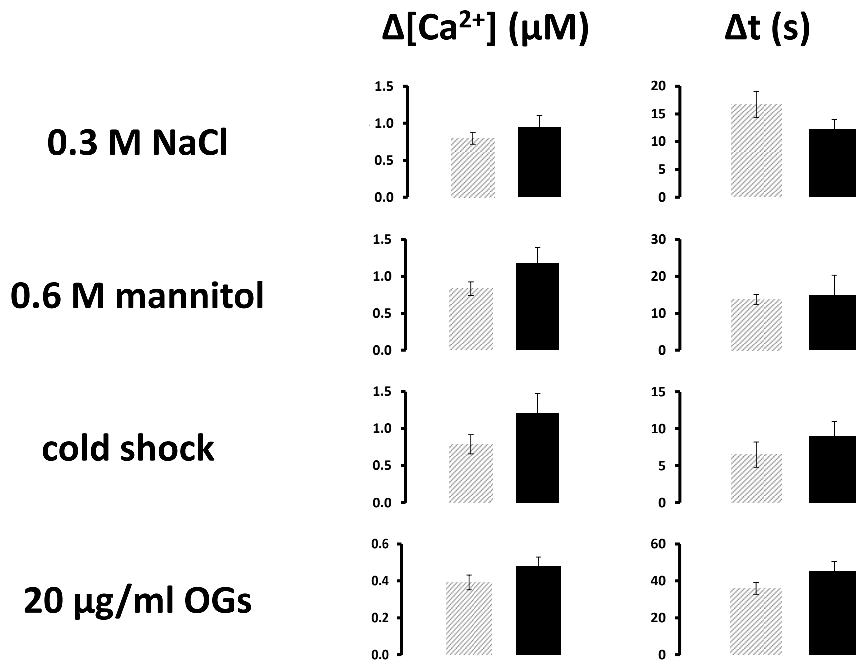
Fig. 4. Set up of *A. thaliana* photoautotrophic cell suspension cultures stably expressing YFP-aequorin in the stroma of chloroplasts (B), starting from the corresponding heterotrophic cell cultures (A). Confocal microscopy analysis demonstrated the presence of much more evident stromules, stroma-filled tubules originating from the plastid surface (white arrowheads), in the amyloplasts of heterotrophic cell cultures (A) than in the chloroplasts of autotrophic cell cultures (B). Bar, 20 µm.

To analyse the potentially different involvement of amyloplasts and chloroplasts in Ca^{2+} homeostasis and signalling, autotrophic cell cultures stably expressing stromal aequorin were challenged with the same stimuli used in the treatments described above for

heterotrophic cell cultures. The results did not highlight significant differences in the Ca^{2+} dynamics recorded in the stroma of non-green *versus* green plastids, concerning either the amplitude or the timing of the maximal Ca^{2+} peak (Supplementary Fig. S2 and Supplementary Fig. S3). However, a clear-cut difference in their Ca^{2+} response emerged when a stimulus more closely related to the specific physiology of the chloroplast was tested, *i.e.* the light-to-dark transition at the end of the 16 h light photoperiod. In this latter case, a transient $[Ca^{2+}]$ elevation was detected in the stroma of chloroplasts, peaking at ~0.44 µM about 20 min after light switch-off and slowly decreasing back to basal values within 2 h (Fig. 5, black trace). By contrast, no $[Ca^{2+}]$ changes were observed in the stroma of amyloplasts (Fig. 5, grey trace).



Supplementary Fig. S2. Comparison between stromal Ca^{2+} dynamics in amyloplasts and dark-adapted chloroplasts after the perception of environmental stimuli. *A. thaliana* heterotrophic (grey trace) and autotrophic (black trace) cell suspension cultures containing amyloplasts and chloroplasts, respectively, and stably expressing stroma-targeted aequorin were challenged (after 100 s, arrow) with different abiotic and biotic stimuli as specified in each panel. Ca^{2+} traces are representative of at least three independent experiments giving similar results.



Supplementary Fig. S3. Statistical analysis of $\Delta[Ca^{2+}]$ and Δt of Ca^{2+} peaks referred to Ca^{2+} dynamics recorded in the plastid stroma of *A. thaliana* heterotrophic and dark-adapted autotrophic cell suspension cultures in response to different environmental stimuli. No significant differences in either $\Delta[Ca^{2+}]$ or Δt of the Ca^{2+} peaks were observed between the two functional types of plastids.

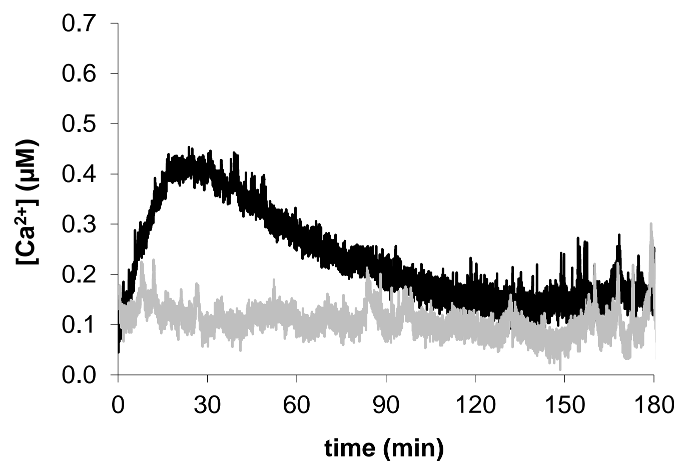


Fig. 5. Effect of light-to-dark transition on plastid stromal $[Ca^{2+}]$ in *A. thaliana* autotrophic and heterotrophic cell suspension cultures. A dark-induced Ca^{2+} transient was observed in the stroma of chloroplasts (black trace) but not amyloplasts (grey trace).

In order to check whether chloroplasts may display differential Ca^{2+} responses also to other stimuli as a function of their metabolic state, Ca^{2+} dynamics were compared between autotrophic cell cultures directly after exposure to 8 h dark and after reactivation of photosynthesis by 16 h light exposure. Light exposure did not alter the overall stromal Ca^{2+} response to either oxidative stress, cold shock, or OGs (Fig. 6). However, a significantly higher $[Ca^{2+}]$ increase was recorded in the stroma of light-reactivated

chloroplasts, when compared to the non-reactivated ones, in response to salt stress (1.53 ± 0.28 µM, n=3, *vs.* 0.71 ± 0.09 µM, n=4) and drought (2.41 ± 0.27 µM, n=4, *vs.* 0.97 ± 0.10 µM, n=4). These results indicate that, although green plastids displayed Ca^{2+} responses that are undistinguishable from those of non-green plastids when their photosynthetic metabolism is switched off by dark exposure (Supplementary Fig. S2 and Supplementary Fig. S3), clear differences emerged after their photosynthetically active status was reactivated by 16 h light exposure (Fig. 6). The effective reactivation of photosynthesis, leading to starch biosynthesis after CO_2 assimilation into photosynthates, was confirmed by the presence of Lugol-stained starch granules in the stroma of autotrophic suspension-cultured cells after 16 h light, but not after 8 h dark (Supplementary Fig. S4).

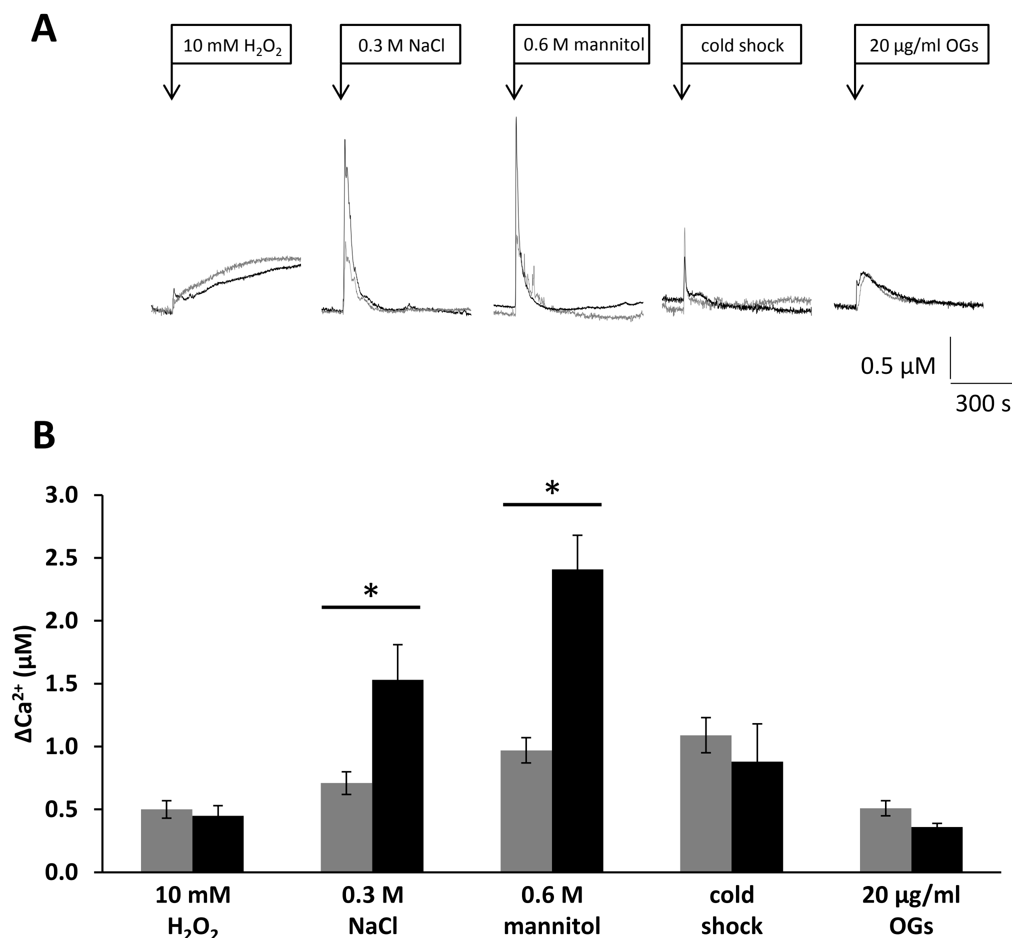
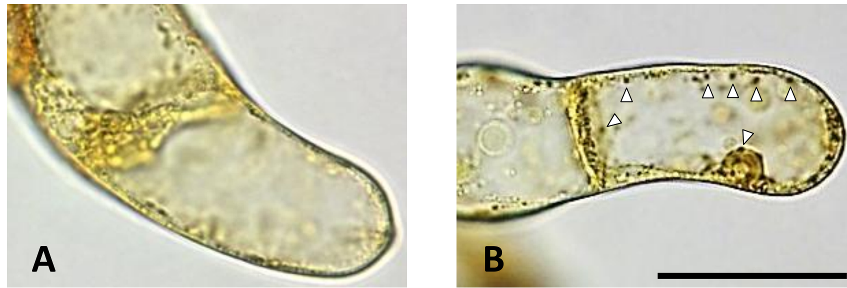


Fig. 6. Comparison of Ca^{2+} dynamics in *A. thaliana* autotrophic cell suspension cultures expressing aequorin in the chloroplast stroma, after exposure to 8 h dark (A, grey traces; B, grey columns) or 16 h light to reactivate the photosynthetic metabolism (A, black traces; B, black columns). Suspension-cultured cells were challenged with the environmental stimuli indicated at the bottom of the columns. In B, statistically significant differences ($p<0.05$) in the $\Delta[Ca^{2+}]_{max}$ are indicated by an asterisk.



Supplementary Fig. S4. Reactivation of photosynthetic metabolism by 16 h-light exposure in *A. thaliana* autotrophic cell cultures. Lugol staining was used to detect primary starch granules (white arrowheads) in chloroplasts, whose number was greatly reduced in suspension-cultured cells at the end of the 8 h dark phase (A) with respect to the end of the 16 h light phase (B). Bar, 50 µm.

Discussion

In order to dissect the involvement of chloroplasts and non-green plastids in Ca^{2+} handling during signal transduction events, we monitored $[Ca^{2+}]$ changes in response to different environmental stimuli in *Arabidopsis thaliana* heterotrophic and autotrophic cell suspension cultures stably expressing aequorin targeted to the organelle stroma. The results presented in this work provide evidence for the ability of amyloplasts, *i.e.* non-green plastids specialized in the accumulation of starch, to undergo a tight regulation of $[Ca^{2+}]$ inside the organelle and to evoke specific Ca^{2+} changes in response to several environmental cues. It is known that Ca^{2+} plays essential regulating roles in the physiology of plastids, by affecting diverse processes, not necessarily correlated only to photosynthesis, such as plastid division and the import of nucleus-encoded proteins (Rocha and Vothknecht, 2012; Nomura and Shiina, 2014; Stael *et al.*, 2012; Hochmal *et al.*, 2015). Nevertheless, a comparison between non-green plastids and chloroplasts in terms of Ca^{2+} dynamics in response to environmental stimuli has never been addressed before. On the basis of the peculiar structural and physiological features of the different functional types of plastids (Jarvis and López-Juez, 2013), distinct responses in terms of Ca^{2+} homeostasis and signalling might be expected. Surprisingly, no significant differences were detected in terms of stromal Ca^{2+} dynamics comparing heterotrophic and dark-adapted autotrophic suspension-cultured cells in response to the considered stimuli. The absence of distinct Ca^{2+} signatures in non-green *versus* green plastids suggests that the two functional types of plastids possess similar mechanisms allowing for Ca^{2+} fluxes in plastids. In resting conditions, $[Ca^{2+}]$ in the cytosol and in the amyloplast stroma was found to be very similar. In response to salt, drought, and oxidative stresses, the timing of the Ca^{2+} peaks recorded in

the different subcellular locations (*i.e.* the bulk cytosol, the plastid outer envelope and the stroma) turned out to be gradually delayed from the cytosol moving towards the plastid stroma, indicating a likely crucial role for plastids in shaping and switching off intracellular Ca^{2+} signals. The kinetics of the observed Ca^{2+} changes, *i.e.* the fact that a decrease in [Ca^{2+}] at the cytosolic surface of the outer membrane of the envelope often correlates with an increase of [Ca^{2+}] in the stroma, support the notion that under these conditions plastids may function as Ca^{2+} sinks, used by the cell to dissipate cytosolic [Ca^{2+}] signals, thus contributing to the modulation of intracellular Ca^{2+} signatures.

In view of the importance of the light stimulus for the physiology of plastids, the effect of light-to-dark transition on stromal Ca^{2+} levels in chloroplasts and non-green plastids, respectively, was evaluated. Previous studies have demonstrated that a long-lasting transient [Ca^{2+}] elevation is detected in the chloroplast stroma after a lights-off stimulus (Sai and Johnson, 2002) and further investigations proved that this elevation is restricted to the organelle and does not involve the cytosol (Nomura *et al.*, 2012). Our aequorin-based Ca^{2+} measurement experiments, carried out in heterotrophic and autotrophic suspension-cultured cells containing amyloplasts or chloroplasts, respectively, allowed for a dissection of the potential differential response of these two functional types of plastids to light-to-dark transition. Our data showed that the dark-induced Ca^{2+} transient occurred in green plastids only, suggesting a potential role of the thylakoid system (exclusively present in chloroplasts) in generating the stromal Ca^{2+} signal. A likely possibility is that the observed stromal Ca^{2+} fluxes derive from the thylakoid lumen, which may act as a mobilizable Ca^{2+} store. Alternatively, complexed Ca^{2+} may also be released from the thylakoid membrane, without the involvement of Ca^{2+} flux-permitting transporters localized at the thylakoid membrane. Indeed, there is a plethora of Ca^{2+}-binding proteins in the chloroplast (Rocha and Vothknecht, 2012; Stael *et al.*, 2012) and upon the light-to-dark transition, these proteins may release Ca^{2+} in the surrounding environment. Moreover, the calcium-sensing receptor CAS, a low-affinity, high-capacity Ca^{2+}-binding protein localized in thylakoid membranes, may play a role as a major actor in this process (Nomura *et al.*, 2008; Vainonen *et al.*, 2008; Weinl *et al.*, 2008). Indeed, the dark-induced stromal Ca^{2+} transient was found to be markedly reduced in a *CAS* knockout background (Nomura *et al.*, 2012). Accurate estimates of [Ca^{2+}] and its changes inside and around the thylakoid membrane system are urgently needed to shed light on the origin of these intraplastidal Ca^{2+} fluxes.

The finding of a stromal Ca^{2+} increase occurring shortly after light-to-dark transition and restricted to chloroplasts prompted us to analyse in more detail the potential light dependency of chloroplast Ca^{2+} responses to environmental cues. Autotrophic cell suspension cultures were exposed to a 16 h light period to allow for the reactivation of the photosynthetic metabolism and stromal Ca^{2+} dynamics of dark-adapted and light-reactivated cell were compared after the perception of oxidative stress, salinity, drought, cold shock and a condition mimicking a pathogen attack (treatment with OGs). Interestingly, significant differences were observed upon challenge with the two hyperosmotic shocks simulating salinity (0.3 M NaCl) and drought (0.6 M mannitol), respectively, whereby more than a two-fold higher $[Ca^{2+}]$ increase was detected in light-reactivated chloroplasts. Since remarkable changes in the amplitude of the stromal Ca^{2+} responses were observed between light-reactivated and dark-adapted chloroplasts, but not between amyloplasts and dark-adapted chloroplasts, an indirect impact of light on the Ca^{2+}-mediated transduction mechanisms of these stresses is inferred. Indeed, it has been demonstrated that drought and salinity stresses alter the normal homeostasis of cells and cause an increased production of reactive oxygen species (ROS) that can be used by the plant as signalling molecules in retrograde signalling (Miller *et al.*, 2010). As ROS-mediated retrograde signalling requires ROS produced by chloroplasts during the light phase, one may speculate that Ca^{2+}-mediated responses to environmental stimuli might differ in light-reactivated green plastids compared to dark-adapted ones. The relevance of interconnected networks of Ca^{2+} and ROS signals is increasingly emerging in plant systemic signalling (Gilroy *et al.*, 2014).

In conclusion, this work shows that *A. thaliana* cell suspension cultures stably expressing the bioluminescent Ca^{2+} reporter aequorin targeted to the plastid stroma can be used as a versatile experimental tool suitable to analyse potential differences in Ca^{2+} dynamics between chloroplasts and non-green plastids during signal transduction events. The detection of delayed transient stromal Ca^{2+} elevations, often corresponding in temporal terms with the decline of the cytosolic ones, suggests that plastids indeed seem to function as Ca^{2+} stores, involved in the switching off of cytosolic Ca^{2+} rises and thus in the shaping of intracellular Ca^{2+} signals. A more direct role of these intra-plastidial Ca^{2+} fluxes, and of the consequent changes in Ca^{2+} concentrations, in the specific physiology of the organelle, not only in chloroplasts but also in amyloplasts, cannot be ruled out and needs to be investigated in details in the future. Indeed, chloroplast-mediated activation of plant immune signalling, *via* specific Ca^{2+} signals evoked in the chloroplast stroma by the

pathogen-associated molecular patterns (PAMP) flg22 has already been demonstrated in *A. thaliana* (Nomura *et al.*, 2012). Chloroplast Ca^{2+} signatures were also recorded in response to other plant defense elicitors, such as cryptogein and OGs (Manzoor *et al.*, 2012). In this paper the OG-induced stromal Ca^{2+} signal was shown not to be restricted to chloroplasts only, but to occur also in amyloplasts, suggesting a potential role of this functional type of plastids, most abundant in the root, in Ca^{2+}-mediated signalling during pathogenic as well as beneficial plant-microbe interactions (Moscatiello *et al.*, 2012).

Future work should address the important issue of the characterization of different Ca^{2+} transporters localized at plastidial membranes, that have just recently started to be identified (Teardo *et al.*, 2010, 2011, 2015; Hochmal *et al.*, 2015). Moreover, the involvement of stromules as well as additional intimate contact sites of plastids with other intracellular compartments, such as the endoplasmic reticulum (Schattat *et al.*, 2011; Mehrshahi *et al.*, 2013; Brunkard *et al.*, 2015), needs to be elucidated in the context of the potential bidirectional exchange of Ca^{2+} during signalling events.

Additional supplementary data

Supplementary Video S1. Single-cell z-stack series of *A. thaliana* heterotrophic cell suspension cultures stably expressing YFP-aequorin targeted to the plastid stroma. Stromules, dynamic stroma-filled tubules extending from amyloplasts, are well evident.

Supplementary Video S2. Single-cell z-stack series of *A. thaliana* autotrophic cell suspension cultures stably expressing YFP-aequorin targeted to the chloroplast stroma. The yellow signal indicates co-localization of YFP fluorescence and chlorophyll autofluorescence.

Acknowledgments

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CHAPTER 4.

A rapid and efficient method to obtain photosynthetic *Arabidopsis thaliana* cell suspension cultures

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Keywords: *Arabidopsis thaliana*, photosynthetic cell suspension cultures, chloroplasts, phytohormones, photosynthetic efficiency

Abstract
Photosynthetic cell suspension cultures are a useful experimental system to analyse a variety of physiological processes at a subcellular and molecular level. Moreover, they can be used as a biotechnological tool to obtain secondary metabolite products in controlled conditions. On the other hand, photoautotrophic cell cultures are quite difficult to obtain and this process is usually laborious and time-consuming. In this work a novel and rapid method to set up photosynthetic cell suspension cultures from the model plant *Arabidopsis thaliana* is described. Germination of *Arabidopsis* seeds on a sucrose-containing agarized culture medium supplemented with 0.25 µg/ml 6-Benzylaminopurine and 0.5 µg/ml 2,4-dichlorophenoxyacetic acid caused the formation of green calli at the level of seedling hypocotyls. The subsequent transfer of these calli in liquid culture medium containing the same concentrations of phytohormone and gradually decreasing sucrose levels under high light condition (110 µmol photons $m^{-2}s^{-1}$) allowed to obtain rapidly proliferating green cell suspension cultures. These cultures were confirmed to be photosynthetic by means of confocal microscopy, PAM imaging and analysis of the chlorophyll content. This method also allows for the establishment of heterotrophic cell suspension cultures, containing amyloplasts as a functional type of plastids, directly from autotrophic ones, in a faster way than previously described procedures.

Plant cell suspension cultures are widely used as a useful and versatile experimental system to analyse complex plant physiological processes at the cellular and molecular level. By resolving the complexity of an *in toto* plant into its elementary units, suspension-cultured cells often represent a suitable tool to study a wide range of phenomena. This type of experimental system has been shown to be suitable to investigate many aspects of ion transport, secondary metabolite production, gene expression and defence responses (Ebel & Mithöfer, 1998; Hall, 2000; Moscatiello et al., 2006; Wilson et al., 2014). In the field of cell signal transduction, cell suspension cultures have been shown to be an excellent mean to investigate calcium-mediated signalling events, by allowing the detection and amplification of even faint calcium signals, sometimes limited *in vivo* to only a particular tissue or cell type (Mithöfer et al., 1999; Lecourieux et al., 2005; Navazio et al., 2007 a and b). Homogeneous plant cell populations were also used to evaluate the translocating properties of cell-penetrating peptides, *i.e.* short cationic peptides that can be used as nanocarriers for the intracellular delivery of proteins (Zonin et al., 2011). Special types of cell cultures, containing for example specific members of the plastid family, may help to decipher molecular, physiological and metabolic mechanisms more easily and rigorously than an *in toto* system, based on the use of entire seedlings.

Unfortunately, for as-yet unknown reasons, photoautotrophic cell cultures are quite difficult to obtain, and have been developed only for a restrict number of species (Roitsch & Sinha, 2002). The set up of heterotrophic and autotrophic cell suspension cultures has recently made it possible to dissect differential stimulus-specific calcium responses of non-green plastids *versus* chloroplasts (Sello et al., 2016, accepted manuscript; see Chapter 3). Previous studies have already shown the advantages of working with photoautotrophic cell cultures (Roitsch & Sinha, 2002; Hampp et al., 2012). Photosynthetic cell suspension cultures are useful for many laboratory procedures and analyses, such as for example the isolation of protoplasts (*i.e.* plant cells enzymatically deprived of their cell wall) and the isolation of chloroplasts for metabolic studies. They are also ideal tools to easily verify the expression and correct targeting of fluorescently-tagged recombinant proteins (see Chapter 3 and 5). Moreover, an experimental system given by suspension-cultured cells, rapidly proliferating in a controlled environment, offers the possibility to slightly change growth conditions and consider the effect of such modifications or of special treatments on cell physiology and specific metabolic processes.

The methods so far developed are in most cases quite laborious and time-consuming. Indeed, the procedure described by Hampp and colleagues (2012), who for the first time

set up a method to establish photoautotrophic cell cultures of *Arabidopsis thaliana*, took about two years.

In this work we describe a novel rapid method to obtain *Arabidopsis* photosynthetic cell suspension cultures, *i.e.* containing chloroplasts as functional type of plastids. The time interval needed to set up such experimental system is greatly reduced when compared to traditionally used methods to establish photoautotrophic cell cultures (Loyola-Vargas & Vázquez-Flota, 2006; Mustafa et al., 2011; Hampp et al., 2012); moreover, the photosynthetic activity of the obtained suspension-cultured cells was demonstrated to be good, and the photoautotroph status of the cell population was found to be a stable condition.

This method provides a newly-designed procedure to obtain chlorophyll-containing cell suspension cultures from *Arabidopsis* seedlings in a much shorter time frame (about two months instead of two years) than the only previously published method from the same source of plant material (Hampp et al., 2012).

Arabidopsis thaliana ecotype Columbia (Col-0) was used to set-up a method for the production of photoautotrophic cell suspension cultures. *Arabidopsis* seeds were surface sterilized for 60 seconds in a 70% ethanol, 0.05% Triton-X 100 solution, 60 seconds in 100% ethanol and let dry on an autoclaved Whatman paper disc for at least 10 minutes. Seeds were sown on MS medium added with 3% sucrose (Duchefa Biochemie, Haarlem, The Netherlands), 0.25 µg/ml 6-Benzylaminopurine (6-BaP), 0.5 µg/ml 2,4-dichlorophenoxyacetic acid (2,4-D), and they were placed in a growing chamber at 24°C with a 16 h /8 h light/dark cycle. These hormones are usually used to grow and maintain heterotrophic cell suspension cultures, as 6-BaP is a synthetic cytokinin that elicits cell division and 2,4-D is a synthetic auxin that promotes cell growth. We observed that, already after one week from sowing, the growth of seedlings was slowed down and they subsequently started to form a green mass of dedifferentiated cells at the hypocotyl level (Fig. 1a). Developing green calli (Fig. 1b) were observed at a Leica MZ16F fluorescence stereomicroscope using the chlorophyll filter (excitation at 460/500 nm and emission 605 nm long pass) for a preliminary chlorophyll-autofluorescence analysis. Images were acquired with the digital camera Leica DFC 480, using the Leica Application Suite (LAS) software.

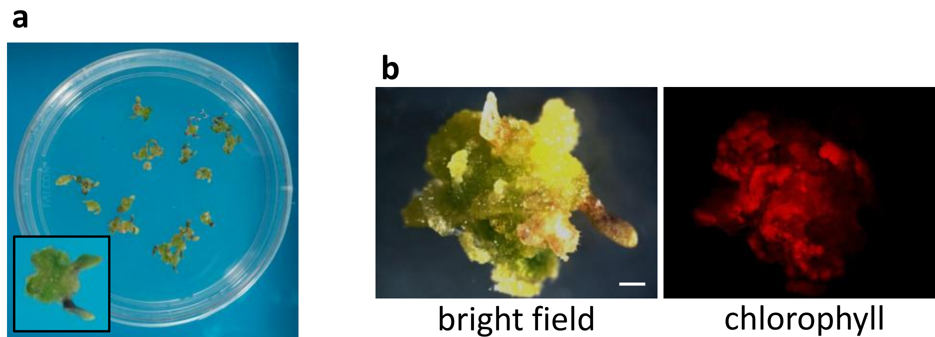

Fig. 1 Exogenous hormone-induced production of green calli from *Arabidopsis* hypocotyls. **a** Surface-sterilized seeds were placed on a cytokynin- and auxin-enriched agarized solid medium containing sucrose (MS, 0.8% agar, 3% sucrose, 0.5 mg/l 2,4-D, 0.25 mg/l 6-BaP) that had a delaying effect on seedling growth and development. Insert: a well-developed callus formed by de-differentiation and proliferations of cells of the hypocotyl after three weeks of germination. **b** Observations at the fluorescence stereomicroscope of green calli, displaying red autofluorescence of chlorophyll when excited with blue light. Bar, 1 mm.

After three weeks well-developed calli were axenically separated from the root and cotyledons, cut in small pieces with a sterilized sharp blade, and transferred into liquid MS medium containing 3% sucrose, 0.5 µg/ml 2,4-D, 0.25 µg/ml 6-BaP to obtain cell suspension cultures (Fig. 2a) (Moscatiello et al., 2013). Erlenmeyer flasks were placed on a shaker at 80 rpm at 23°C under relatively high light conditions (90-110 µmol photons $m^{-2}s^{-1}$) and renewed every week transferring 1 packed cell volume (PCV) of cell culture in 20 ml of MS medium containing stepwise less sucrose to reach 0.5%, in order to stimulate photosynthetic activity (Hampp et al., 2012). In particular, sucrose concentration was gradually reduced from 2% - 1% - 0.5% every two-three weeks (Fig. 2a). Once stabilized in the lowest sucrose concentration condition, a growth curve for the newly established photosynthetic culture cell line was determined (Fig. 2b). Aliquots of suspension-cultured cells were collected at 0, 2, 4, 6, 8, 10 and 12 days, and the fresh weight was determined as described by Moscatiello et al. (2013). The cell suspension culture was found to increase five times in its fresh weight in eight days, following a phase of exponential rate of growth. After 8 days from sub-culturing, a gradual decrease in fresh cell weight was observed, indicative of partial cell lysis (Fig. 2b).

Samples of 5-day old cell suspension cultures were analysed using a Leica TCS SP5 II confocal laser scanning system mounted on a Leica DMI6000 inverted microscope. Excitation was carried out with an Ar laser at 488 nm and the emitted autofluorescence of chlorophyll was detected at 680/720 nm. Confocal microscopy analyses of the suspension-cultured cells confirmed the presence of chloroplasts as functional type of plastids in the newly established green cultures (Fig. 2c).

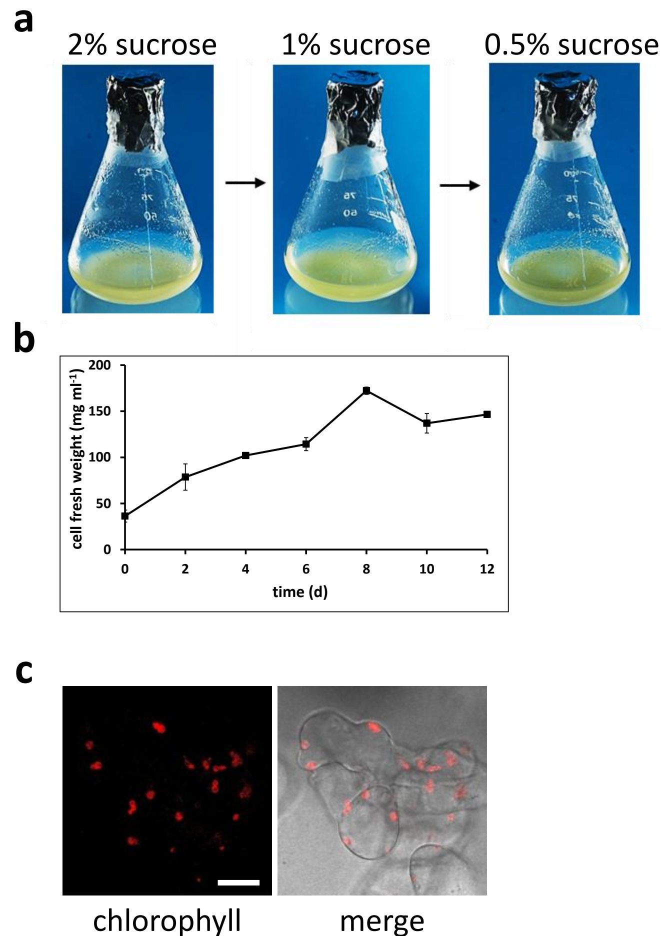

Fig. 2 Set up of *Arabidopsis* photosynthetic cell suspension cultures, exposed to a relatively high illumination rate (110 µmol photons m^{-2} s^{-1}) and maintained in MS culture media containing 0.5 µg/ml 2,4-D and 0.25 µg/ml 6-BaP as hormones, and gradually reduced sucrose concentrations to stimulate the photosynthetic activity. **a** Growth curve of the photosynthetic cell suspension culture, after 3 sub-culturing steps in 0.5 % sucrose containing medium. Data are the means ± s.d. of three independent replicates for each time points. **c** Confocal microscopy analysis confirmed the presence of chloroplasts as functional type of plastids in the cell suspension cultures. Bar, 20 µm.

In order to check whether chloroplasts were effectively photosynthetically active, autotrophic cell cultures were subjected to Pulse Amplitude Modulation (PAM) analyses. 5 days-old suspension-cultured cells were placed in a Closed FluorCam 800 MF (Photon Systems Instruments, Drasov, Czech Republic) and the maximal photosynthetic efficiency of photosystem II (F_V/F_M, where F_V is the variable fluorescence given by basal fluorescence subtracted to maximal fluorescence, and F_M is the maximal fluorescence) was analysed and recorded. The cell cultures under investigation were found to have a good photosynthetic efficiency, since the F_V/F_M value was 0.53 (where 0 is the minimum and 1 is the maximum) (Fig. 3). PAM analyses give an indication about the maximal activity that can be potentially reached only by chlorophylls of photosystem II, as the pulse of light

used for the measurement is saturating, but does not last enough to activate the full photosynthetic process.

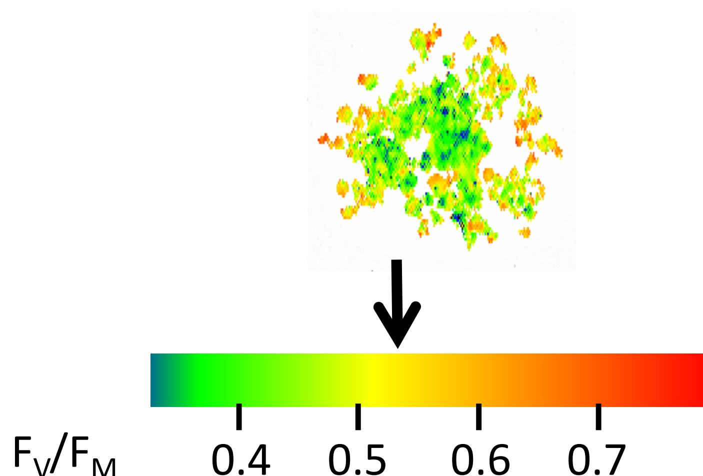

Fig. 3 Photosynthetic activity of *Arabidopsis* autotrophic cell suspension cultures. PAM imaging analysis confirmed that photosystem II had a good maximal potential photosynthetic activity. Black arrow indicates a F_V/F_M mean value of 0.53 (n=3).

Further analyses were also carried out, in order to verify the total photosynthetic pigment content of the cell suspension cultures. Total chlorophylls and carotenoids were extracted in 80% acetone. Suspension-cultured cells (2 ml) in the exponential phase of the growth curve were pelletted down by centrifugation (1600 rpm for 2 min), the culture medium was removed and the fresh weight annotated. Acetone (500 µl) was added in the tubes and the samples were incubated at 4°C for 48 h in the dark. Samples were then centrifuged, the supernatant was transferred in new tubes and stored at -20°C. Pigments were quantified at the spectrophotometer by reading the absorbance at 664 and 646 nm for chlorophyll *a* and *b*, and at 470 nm for carotenoids. Pigments concentration was calculated following equations from Wellburn (1994) and referred to g of fresh weight (Tab. 1).

Tab. 1 Content of photosynthetic pigments in *Arabidopsis* photosynthetic cell suspension cultures. Data are presented as means ± S.E (n=4).

chlorophyll *a* / fresh weight (µg/g)	chlorophyll *b* / fresh weight (µg/g)	chlorophyll *a+b* / fresh weight (µg/g)	Carotenoids / fresh weight (µg/g)
4.13 ± 0.12	2.47 ± 0.46	6.60 ± 0.56	3.08 ± 0.52

Although the obtained *Arabidopsis* cell suspension culture was found to have a good PSII potential photosynthetic efficiency (Fig. 3), the total chlorophyll content, expressed as chlorophyll/fresh weight ratio, was found to be at lower levels than in previously published

works (Huesemann et al., 1979; Katoh et al., 1979; LaRosa et al., 1984). In a previously described procedure, two-tiered flasks with a carbonate buffer in the lower compartment were used to maintain about 2% CO_2 in the atmosphere (Hampp et al., 2012). Growth conditions were different in our experimental system, *i.e.* cell suspension cultures were maintained in sterile Erlenmeyer flasks containing one-fifth volume of culture medium to ensure a proper aeration of the cell culture. Therefore, it can be possible that the low concentration of pigments in our *in vitro* culture system may be due to a reduced rate of inorganic carbon availability. Further experiments will be needed to improve the experimental set up by providing an additional source of CO_2 in the culturing system, thus increasing the content of photosynthetic pigments of the cell cultures. However, results obtained with the PAM imaging (Fig. 3) attested the good level of photosynthetic efficiency of the cell cultures, suggesting their suitability for photosynthetic, metabolic and signalling studies.

This new method was found to be useful not only to rapidly obtain photoautotrophic cell suspension cultures, but also heterotrophic ones. Indeed, classical methods to set up in vitro cell cultures traditionally rely on the application of a callus induction medium (CIM) to make hypocotyls and cotyledons de-differentiate and generate calli, *i.e.* clusters of dedifferentiated cells proliferating on the surface of solid culture media. The complete procedure necessary to obtain rapidly proliferating cell suspension cultures containing amyloplasts as functional type of plastids involves a time-consuming procedure consisting of many different *in vitro* steps (Loyola-Vargas & Vázquez-Flota, 2006; Moscatiello et al., 2013). By applying our novel protocol, we were able to set up heterotrophic cell suspension cultures directly from photoautotrophic ones, thus significantly shortening the required time interval. After obtaining photosynthetic cell suspension cultures, they were subcultured into MS medium, containing 0.5 μg/ml 2,4-D, 0.25 μg/ml 6-BaP, and supplemented with a high concentration of organic carbon (3% sucrose). Moreover, flasks were wrapped in aluminium foil and placed on a shaker at 80 rpm at 24°C. After 2÷3 weeks, cells completely lost their ability to photosynthesize and plastids effectively turned from chloroplasts to amyloplasts, allowing for the obtainment of functional heterotrophic cell suspension cultures. The identity of plastids as amyloplasts was ascertained by microscopy observations, that highlighted the total lack of chlorophyll autofluorescence (Fig. 4a), together with a remarkable level of Lugol-stained starch granules inside these organelles (Fig. 4b).

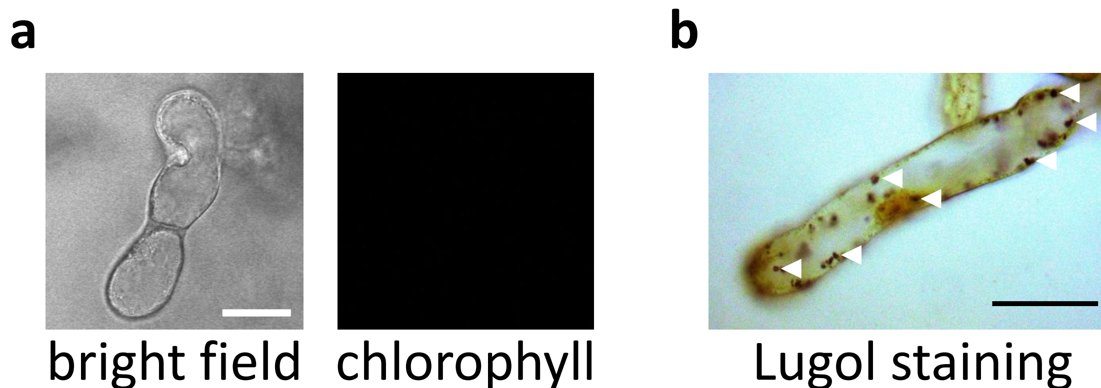

Fig. 4 Set up of heterotrophic cell suspension cultures, starting from the previously established photosynthetic cultures. Microscopy observations of suspension-cultured cells, demonstrating the lack of any chlorophyll fluorescence signal (**a**) as well as the presence of well-evident Lugol-stained starch granules (**b**) (white arrows). Bars, 25 µm (**a**), 50 µm (**b**).

In conclusion, in this work a novel procedure to rapidly obtain photosynthetic *Arabidopsis* cell suspension cultures containing chloroplasts as functional type of plastids is presented. In comparison with previously published methods (Mustafa et al., 2011; Hampp et al., 2012), the time frame needed to set up photoautotrophic cultures is highly reduced (about two months *versus* two years). These photosynthetic *Arabidopsis* cell suspension cultures may also be useful as plant material source for the isolation of protoplasts, that can be used as a valid alternative to *Arabidopsis* leaves. Indeed, protoplast isolation from cell suspension cultures is a quick, highly reproducible and straightforward procedure (Moscatiello et al., 2013) that typically gives high yields (from 10^5 to 10^6 protoplasts / ml of exponentially growing suspension-cultured cells). Similarly, intact chloroplasts can be directly isolated from cell suspension cultures, homogeneous cell populations that can be available every week in bulk quantities and with very reproducible conditions. Moreover, a diverse array of experiments can be conveniently performed in photosynthetic suspension-cultured cells, that virtually contain chloroplasts as unique type of plastids. For example, photoautotrophic cell cultures stably expressing aequorin chimeras targeted to the thylakoid membrane and thylakoid lumen were found to be an ideal experimental system to carry out Ca^{2+} measurement assays aimed at monitoring the concentration of Ca^{2+} and its changes in different chloroplast subcompartments (Sello et al., unpublished; see Chapter 5). An additional advantage of this method is that, by simply transferring the photosynthetic cultures from a 16 h light /8 h dark photoperiod to a condition of total darkness, in a culture medium containing a high concentration of sucrose

(3%) as source of organic carbon, heterotrophic cell suspension cultures can be easily and rapidly obtained.

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CHAPTER 5.

Targeting of aequorin chimeras to the thylakoid system uncovers intra-chloroplast calcium fluxes triggered by environmental cues in *Arabidopsis thaliana*

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Key words: aequorin, *Arabidopsis*, calcium, environmental cues, light-to-dark transition, thylakoid lumen, thylakoid membrane.

Summary

- Although it is known that calcium regulates photosynthesis and other chloroplast-specific processes, knowledge about the participation of the different chloroplast sub-compartments in Ca^{2+} homeostasis and signalling is still in its infancy. The aim of this work was to investigate the involvement of the thylakoid system in Ca^{2+} handling and in the modulation of chloroplast Ca^{2+} signals.
- *Arabidopsis thaliana* lines stably expressing the Ca^{2+}-sensitive photoprotein aequorin targeted to the thylakoid lumen and thylakoid membrane were established. After verification of the correct localization of the bioluminescent Ca^{2+} reporter, they were used in Ca^{2+} measurement assays in response to different environmental stimuli.
- In resting conditions, [Ca^{2+}] was assessed to be about 0.5 μM in the thylakoid lumen, *i.e.* 3-to-5 fold higher than in the chloroplast stroma; moreover, Ca^{2+} changes in the thylakoid lumen were found to be triggered by abiotic cues, including light-to-dark transition. In some cases microdomains of high [Ca^{2+}] were recorded at the surface of the thylakoid membrane, closely mirroring transient Ca^{2+} changes occurring in the bulk stroma.
- These aequorin chimeras targeted to the thylakoid system may serve as a valuable tool for future studies on organellar Ca^{2+} signalling aimed at elucidating the integration of chloroplasts in the complex plant calcium signalling network.

Introduction

Calcium is a universal signalling element involved in a wide range of physiological processes in all eukaryotes. Although there are common elements in Ca^{2+}-based signal transduction networks, unique features of plant Ca^{2+} homeostasis and signalling derive from both structural features of plant cells and from differences in the lifestyle and developmental programs of plants. Ca^{2+} plays a well-known pivotal role in transducing environmental signals in plants: rapid changes in intracellular free Ca^{2+} concentration are evoked by a wide variety of abiotic and biotic stimuli (Dodd et al., 2010). Despite almost every intracellular compartment is able to maintain a proper Ca^{2+} concentration and potentially mobilize the ion (Stael et al., 2012), the precise contribution of each of them in the complex plant Ca^{2+} signalling network has not yet been analysed in detail. The vacuole is traditionally considered as the main intracellular Ca^{2+} reservoir of the plant cell, due to its prominent role in the overall ion homeostasis and remarkable volume within mature plant cells. Its involvement as a major intracellular stimulus-releasable Ca^{2+} store has been demonstrated in the signal transduction pathways triggered by different abiotic stress, *e.g.* cold shock, drought and salinity (Knight et al. 1996 and 1997). Moreover, Ca^{2+} release from the vacuole mediated by the vacuolar cation channel TPC1 has been suggested to be involved in systemic signalling activated upon salt stress (Choi et al., 2014), wounding and herbivory (Kiep et al., 2015).

In the last few years there has been a growing interest regarding the ability of other plant-specific organelles, *i.e.* chloroplasts, in participating to Ca^{2+} homeostasis and signalling. Although total Ca^{2+} concentration in chloroplasts has long been assessed to be as high as 15÷25 mM (Larkum, 1968; Yamagishi et al., 1981), free Ca^{2+} concentration in the chloroplast stroma was found to be much lower, *i.e.* about 150 nM (Johnson et al., 1995). A large body of evidence indicates that chloroplasts have a need for a tight, fine-tuned control of the organellar concentration of this ion. Indeed, Ca^{2+} is involved in the control of several metabolic processes in chloroplasts, as it is required as a cofactor for the evolution of oxygen carried out by the oxygen-evolving complex, it regulates the activity of some enzymes involved in CO_2 fixation (*i.e.* fructose-1,6-bisphosphatase and sedoheptulose-l,7-biphosphatase) and it affects the import of nuclear-encoded proteins into the organelle via the translocon (TOC/TIC) complexes at the outer/inner envelope of chloroplasts (Rocha & Vothknecht, 2012). Targeting of the bioluminescent Ca^{2+} reporter aequorin to the chloroplast stroma highlighted the occurrence of circadian oscillations in

chloroplast free Ca^{2+} (Johnson et al., 1995), and the induction of stromal Ca^{2+} fluxes upon light-to-dark transition (Sai & Johnson, 2002). The potential contribution of chloroplasts to originating cytosolic Ca^{2+} signals has recently come to the fore by the identification of the Ca^{2+}-sensing receptor CAS: this plant-specific protein, localized to thylakoid membranes, was found to control cytosolic [Ca^{2+}] transients induced by external Ca^{2+} and subsequent stomatal closure, by regulating chloroplast Ca^{2+} storage/mobilization through a still unknown mechanism (Nomura et al., 2008; Weinl et al., 2008). In addition to a potential role in the modulation of cytosolic Ca^{2+} signals, emerging evidence suggests that also Ca^{2+} signals within the chloroplasts may equally play essential roles in the plant cell signalling network. Indeed, CAS-mediated stromal Ca^{2+} transients were shown to be responsible for the activation of the plant immune system in *Arabidopsis* (Nomura et al., 2012).

To better understand how chloroplasts are involved in intracellular Ca^{2+} handling, it is necessary to be able to monitor in a sensitive and accurate way Ca^{2+} dynamics in the different organellar subcompartments. Constructs encoding the bioluminescent Ca^{2+} reporter aequorin targeted to the outer and inner surface of the chloroplasts, in addition to the stroma, have recently been engineered (Mehlmer et al., 2012). Nevertheless, information about [Ca^{2+}] and its changes during signal transduction events in the thylakoid system is so far missing. In this work YFP-fused aequorin probes targeted to the thylakoid lumen and the thylakoid membrane, facing the stroma, were generated. *Arabidopsis* lines stably expressing this novel set of aequorin chimeras were used in Ca^{2+} measurement assays in response to different abiotic and biotic stimuli. Monitoring of chloroplast Ca^{2+} dynamics revealed the occurrence of stimulus-specific Ca^{2+} signals, characterized by unique kinetic parameters, in the different chloroplast sub-locations. In particular, evidence was provided for dark-stimulated intra-chloroplast Ca^{2+} fluxes, which suggested a new scenario for organellar Ca^{2+} transport upon light-to-dark transition.

Materials and methods

Chemicals

All chemicals, if not specified, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Molecular cloning and construction of expression plasmids

In the constructs used for the generation of *Arabidopsis* transgenic plant lines stably expressing aequorin targeted to the thylakoid membrane and the thylakoid lumen, the coding sequence of aequorin was fused to the first 92 amino acids of the THYLAKOID LUMEN PROTEIN 18.3 (TLP) (At1G54780.1) and the first 137 amino acids (corresponding to the N-terminal domain) of the thylakoid localized State Transition protein kinase 7 (STN7) (At1G68830). First, these targeting sequences were cloned into vectors carrying the YFP-aequorin fusion protein (YA) in order to create an expression cassette in which the expression is driven by the UBI10 promoter. Afterwards, the whole expression cassette (Fig. 1) of the different constructs were cloned into pBIN19 vectors carrying the Basta resistance via the restriction sites *KpnI* and *SpeI*.

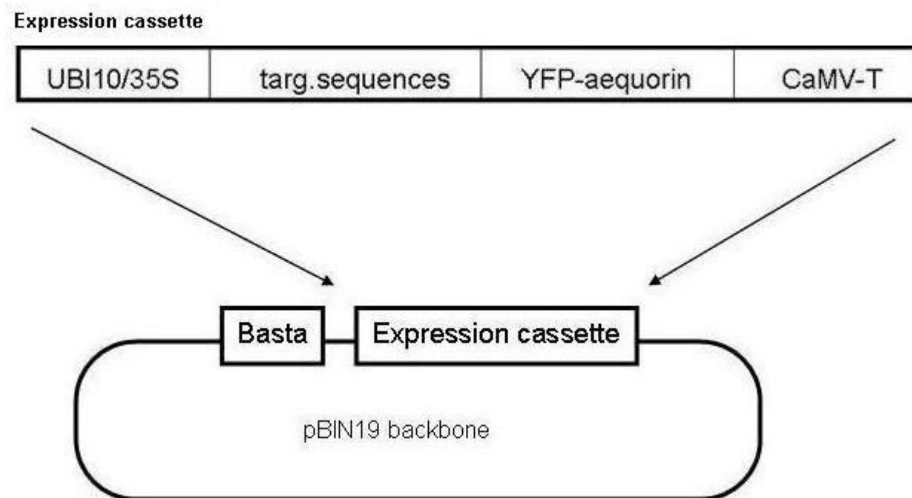

Fig. 1 Cloning strategy for the creation of binary expression vectors targeting YA to different cellular compartments. All the targeting sequences were cloned into an expression cassette in front of YA. The complete expression cassette was afterwards transferred into the binary vector pBIN19 (Basta) (from Mehlmer et al., 2012).

Table 1 shows the amino acid sequences used for the targeting of the two YFP-aequorin fusion constructs to the thylakoid system.

Table 1. Amino acid sequences which were fused to YFP-aequorin to generate expression vectors encoding aequorin chimeras targeted to the thylakoid lumen and thylakoid membrane, respectively.

Name	Amino acid sequence	Subcellular localization
TLP	METLLSPRALSPPLNPKPLSLHQTKPTS HSLSLSKPTTFSGPKHLSTRFTKPESRN WLIDAKQGLAALALSLTLTFSPVGTAL ASEFNILND	**Thylakoid lumen**
STN7	MATISPGGAYIGTPSPFLGKKLKPFSLT SPILSFKPTVKLNSSCRAQLIDTVHNLFI GVGVGLPCTVMECGDMIYRSTLPKSN GLTITAPGVALALTALSYLWATPGVAP GFFDMFVLAFVERLFRPTFRKDDFVVG	**Thylakoid surface exposed to the stroma**

***Agrobacterium tumefaciens* transformation**

In an ice-chilled electroporation cuvette 50 µl of competent *A. tumefaciens* cells (GV3101) were mixed with 2 µl of plasmid DNA and electroporated at 1.6 kV. Before plating, the cells were diluted with 200 µl LB medium. GV3101 was plated on LB medium containing 50 µg/ml kanamycin, and 25 µg/ml gentamicin. Bacterial cells were grown for 3 days at 28°C.

***Arabidopsis* transformation**

Arabidopsis thaliana plants ecotype Columbia (Col-0) were transformed with the constructs encoding aequorin probes targeted to the thylakoid lumen and thylakoid membrane by using the floral dip method, as described by Clough and Bent (1998). Briefly, 200 mL LB media containing 50 µg/ml kanamycin and 25 µg/ml gentamicin were inoculated with the appropriate GV3101 *Agrobacterium* strain containing the pBIN construct. The bacteria were incubated overnight at 28°C. After centrifugation, the bacterial pellet ($OD_{600\ nm}$ = 0.8) was resuspended in 5% sucrose and 0.025% Silwet-L77 was added. The already formed siliques of *Arabidopsis* plants were removed and the inflorescences of the plants were dipped into the bacteria suspension. Afterwards, the whole plants were wrapped into plastic bags and incubated in the dark overnight. On the next day, the bags were removed and plants were placed back into the growing chamber until the seeds were matured. In order to increase the transformation efficiency, the

procedure was repeated after 7 days. Primary T1 transformants were identified by Basta (50 µM) selection on soil. The selected plants were transferred into single pots and watered regularly until the siliques had fully developed. Afterwards they were transferred to a drying room until dried to a sufficient level for seed collection.

Aequorin expression analyses

RT-PCR analyses were carried out using *Arabidopsis* leaves from 1-month old Basta-resistant plants. Total RNA was extracted using the RNeasy Plant Mini Kit (Quiagen) and reverse transcribed with SuperScript® III (Invitrogen) according to manufacturer's instructions. Primers designed on the cDNA sequence of aequorin (forward 5'-tcgacaacccaagatggattgga-3'; reverse 5'-tgatagcatgcgaattcatcagtgttttat-3') were used to analyze aequorin gene expression. The coding sequence of actin was used as housekeeping gene (forward: 5'-ggttgcaccgccagagagaaaatac-3'; reverse 5'-aacaaactcaccaccacgaaccaga-3').

Immunoblot analyses were carried out on total protein extracts obtained from leaves of 1-month old *Arabidopsis* plants, as previously described (Zonin et al., 2011). Samples were separated on 10% SDS-PAGE and gels were blotted on PVDF membranes (Immobilon, Millipore, Darmstad, Germany). Blots were incubated with a polyclonal anti-aequorin antibody (Abcam, Cambridge, UK) diluted 1:10000. As a positive control, an *Arabidopsis* line stably expressing aequorin in the cytosol, excluding the nucleus ($CPK17_{G2A}$-NES-YA), was used (Mehlmer et al., 2012).

Aequorin activity measurements

Sub-lines of the T2 generations obtained from transformation of *Arabidopsis* with the constructs encoding aequorin targeted to the thylakoid lumen (TM line) and thylakoid membrane (TM line) were screened for aequorin activity. Luminescence levels were monitored *in planta* with an aequorin-measuring apparatus (Electron Tubes Limited, Middlesex, UK). *Arabidopsis* seeds were surface sterilized for 60 seconds in a 70% ethanol, 0.05% Triton-X 100 solution, 60 seconds in 100% ethanol and let dry on an autoclaved Whatman paper disc for at least 10 minutes. Seeds were subsequently plated on half-strength Murashige and Skoog (MS) medium (Duchefa Biochemie, Haarlem, The Netherlands) containing 0.8% plant agar, supplemented with 1.5% sucrose and 50 µM Basta. Five 11-to-14 day-old seedlings from all sub-lines were reconstituted overnight with 5 µM coelenterazine (Prolume Ldt, Pinetop, Arizona, USA) and kept at 24°C in the dark.

The day after they were singly placed in the luminometer chamber containing 300 µl H_2O and total aequorin was discharged by injection of an equal volume of 30% ethanol, 1 M $CaCl_2$. Total luminescence values from every sub-line were pooled together and the average luminescence was calculated.

Set up of suspension cell cultures containing chloroplasts from the transformed *Arabidopsis* lines

Autotrophic cell suspension cultures were obtained according to Sello et al. (unpublished; see Chapter 4) using seeds of the T2 generation from the sub-lines stably expressing the aequorin chimeras targeted to the thylakoid lumen and thylakoid membrane that showed the highest aequorin activity. Briefly, *Arabidopsis* seeds of the T2 generation from TL and TM sub-lines that showed the highest aequorin activity were surface sterilized as described above. Seeds were plated on MS medium supplemented with 3% sucrose, 0.25 µg/ml 6-Benzylaminopurine (6-BaP), 0.5 µg/ml 2,4-dichlorophenoxyacetic acid (2,4-D) and 50 µM Basta. After 48 h stratification in darkness at 4°C, MS plates were incubated in a growing chamber at 24°C with a 16/8 h light/dark cycle. After about 3 weeks, green calli developed from hypocotyls. Those showing the higher YFP fluorescence were separated from the root and cotyledons, cut in small pieces with a sterilized sharp blade, and transferred in liquid MS medium containing 2% sucrose, 0.5 µg/ml 2,4-D, 0.25 µg/ml 6-BaP and 50 µM Basta to obtain cell suspension cultures. Cultures were incubated on a shaker at 80 rpm under relatively high light conditions (110 µmol photons $m^{-2}s^{-1}$) and subcultured every week by transferring 1 packed cell volume (PCV) of cell culture in 20 ml of MS medium containing decreasing sucrose concentration (up to 0.5%) to stimulate photosynthetic activity (Hampp et al., 2012).

Fluorescence microscopy analyses

Arabidopsis developing calli were observed under a Leica MZ16F fluorescence stereomicroscope, equipped with a GFP filter (excitation at 450/490 nm and emission at 500/550 nm) and a chlorophyll filter (excitation at 460/500 nm and emission above 605 nm). Images were acquired with the digital camera Leica DFC 480 using the Leica Application Suite (LAS) software.

Photoautotrophic cell suspension cultures were observed under a Leica TCS SP5 II confocal laser scanning system mounted on a Leica DMI6000 inverted microscope with automated programmable scanning stage and motorized lens turret. Excitation with the Ar

laser was carried out at 488 nm and the emitted fluorescence was detected at 505-530 nm for YFP and at 680-720 nm for chlorophyll.

Pulse Amplitude Modulation (PAM) analyses

5-day-old cell suspension cultures were placed in a Closed FluorCam 800 MF (Photon Systems Instruments, Drasov, Czech Republic) and the photosynthetic activity (F_V/F_M, where F_V is the variable fluorescence given by maximal and basal fluorescence, and F_M is the maximal fluorescence) was measured.

Immunocytochemical analyses

Leaf fragments from about 1 month-old *Arabidopsis* plants were fixed overnight at 4°C in 4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M cacodylate buffer (CAB), pH 7.0. After 3 washes in CAB, dehydration in a graded ethanol series was performed. Samples were then progressively embedded in medium-grade London Resin White (LRW, PolyScience). Ultrathin sections (500 Å) were obtained on a Reichert-Jung Ultramicrotome and mounted on uncoated nickel grids. For immunogold labelling, grids were incubated for 45 min in 0.1% Tween 20, 1% BSA in TBS and then for 1 h with the rabbit anti-aequorin antibody at a 1:1000 dilution. After 3 washes with 0.1% Tween 20 in TBS, samples were incubated for 1 h with an anti-rabbit secondary antibody conjugated with colloidal gold particles (10 nm diameter) diluted 1:100. After 2 washes as above and 1 wash in distilled H_2O, samples were exposed to osmium tetroxide vapours overnight. After extensive washing with distilled H_2O, samples were counterstained with uranyl acetate and lead citrate and observed using a Tecnai 12-BT transmission electron microscope (FEI, Eindhoven, The Netherlands) operating at 120 kV equipped with a Tietz camera. *Arabidopsis* wild-type and stably expressing aequorin in the chloroplasts stroma (Mehlmer et al., 2012) were used as negative and positive controls, respectively.

Chloroplast isolation and thermolysin treatment

Chloroplasts were isolated from leaves of 1 month-old *Arabidopsis* plants using the procedure described by Aronsson and Jarvis (2002). Chloroplasts, resuspended in Wash Buffer (50 mM Hepes/KOH, 3 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) were vigorously diluted with 10 volumes of Osmotic Shock Buffer (50 mM Hepes/KOH, 3 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) and then centrifuged at 48000 g for 10 min at 4°C to isolate thylakoids. The thylakoid-containing pellet was then resuspended in Wash Buffer. Treatment with the

proteolytic enzyme thermolysin was carried out as described by Mehlmer et al. (2012). Briefly, the thylakoid suspension (50 μg of protein) was incubated with thermolysin (0.1 μg/μl) for 20 min on ice, and the reaction was stopped by the addition of 5 mM EDTA. Immunoblot analyses were carried out as described above.

Ca^{2+} measurement assays in the *Arabidopsis* cell lines stably expressing aequorin targeted to the thylakoid system

Exponentially-growing (4 day-old) autotrophic cell suspension cultures were reconstituted overnight with 5 μM coelenterazine. On the following day, after extensive washing, 50 μl of cells were placed in the luminometer chamber and challenged with different environmental stimuli, as previously described (Sello et al., 2015, accepted manuscript; see Chapter 3). Oxidative stress (10 mM H_2O_2), salt stress (0.3 M NaCl) and pathogen attack (20 μg/ml oligogalactunonides with a degree of polymerization from 10 to 15) were simulated by the injection in the cell suspension culture of an equal volume of a 2-fold concentrated stimulus dissolved in plant cell culture medium. Ca^{2+} dynamics were recorded for a total of 700 seconds before the injection of 100 μl of the discharge solution (30% ethanol, 1 M $CaCl_2$). Autotrophic cell suspension cultures stably expressing aequorin in the chloroplast stroma, already available in the laboratory (Sello et al., 2015, accepted manuscript; see Chapter 3) were used as a comparison. The light signal was collected and converted off-line into Ca^{2+} concentration values using a computer algorithm based on the Ca^{2+} response curve of aequorin (Brini et al., 1995).

Monitoring of intra-chloroplast Ca^{2+} dynamics in response to light-to-dark transition

Seeds of *Arabidopsis* lines stably expressing aequorin targeted to the cytosol, outer surface of the plastid envelope, stroma (Mehlmer et al., 2012), thylakoid membrane and thylakoid lumen (set up in this work) were surface-sterilized and sown on agarized (0.8% plant agar) half-strength MS medium containing 1.5% sucrose and the appropriate selective agent (50 μg/ml kanamycin or 50 μM Basta) to germinate under a 16 h/8 h light-dark cycle at 24°C. 7 to 14 day-old seedlings were incubated, on the day before the experiment, in 5 μM coelenterazine to allow for the reconstitution of aequorin in the darkness for at least 2 h. At the beginning of the following dark phase, seedlings were quickly transferred into the luminometer chamber containing 300 μl H_2O and Ca^{2+} dynamics were monitored for a total of 3 h. At the end of the experiment, discharge of the

remaining aequorin pool was carried out as described above. In some experiments, seedlings were pre-treated for 30 min with 5 μM nigericin before the end of the light phase. A negative pressure was repeatedly (5 times) applied through a syringe to ensure an efficient internalization of the H^+-transporting ionophore into seedlings.

Results

Generation of constructs encoding YFP-aequorin fusion proteins targeted to the thylakoid lumen and thylakoid membrane

The aim of this work was to generate constructs encoding aequorin probes that may allow for the measurement of [Ca^{2+}] and its changes in the thylakoid system, *i.e.* the thylakoid lumen and the thylakoid membrane. To this purpose, the coding sequence of aequorin was fused to the nucleotide sequences encoding for protein domains that were selected to assist in the targeting process. To allow for an easier verification of the correct subcellular localization of the recombinant proteins, the constructs also contained the coding sequence of YFP, fused to the N-terminus of aequorin. Expression is driven by the UBI10 promoter (Fig. 1). To engineer an aequorin probe targeted to the thylakoid lumen, the nucleotide sequence encoding the first 92 amino acids of TLP (THYLAKOID LUMEN PROTEIN 18.3, At1G54780.1) was fused to the coding sequence of YFP-aequorin. To anchor the Ca^{2+} indicator at the thylakoid surface exposed to the stroma, the N-terminal domain of STN7 (THYLAKOID LOCALIZED STATE TRANSITION KINASE, At1G68830) was fused to the coding sequence of YFP-aequorin. This construct contains the nucleotide sequence encoding for the first 137 amino acids of the precursor protein of STN7 including the chloroplast transit peptide and thylakoid insertion sequence (Table 1). The whole expression cassettes were cloned into the pBIN19 vector under the control of the UBI10 promoter and carrying a Basta resistance cassette (Fig.1), for stable transformation of *Arabidopsis* plants.

Generation of *Arabidopsis* transgenic lines stably expressing the aequorin chimeras targeted to the thylakoid system

Arabidopsis plant lines stably transformed with the constructs encoding the aequorin probes targeted to the thylakoid lumen and thylakoid membrane were generated. RT-PCR analyses and immunoblot analyses on Basta-resistant plants confirmed the expression of

aequorin in all tested *Arabidopsis* lines. Gene expression analysis demonstrated that the aequorin mRNA is present in the plant material, as its cDNA was amplified using the selected primers (Fig. 2a). Immunoblot analysis by using an anti-aequorin antibody on total protein extracts separated by 10% SDS-PAGE confirmed the expression of the Ca^{2+} probe in both the lines transformed with either constructs, as protein bands with the expected molecular weight were highlighted (Fig. 2b). Some representative examples of *Arabidopsis* lines stably expressing aequorin targeted to the thylakoid lumen (TL) or to the thylakoid membrane (TM) are shown in Fig. 2a and 2b.

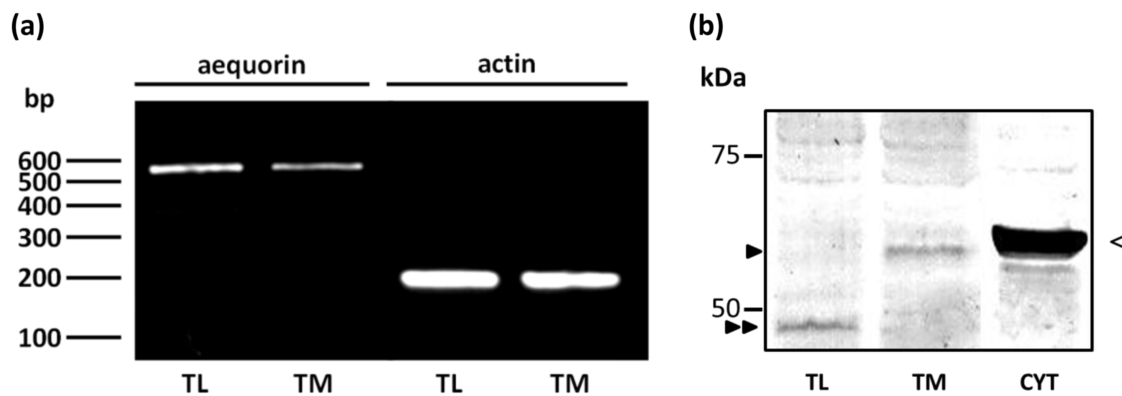

Fig. 2 Analysis of aequorin expression by RT-PCR (a) and immunoblot (b) in *Arabidopsis* transgenic lines stably transformed with the constructs encoding the Ca^{2+}-sensitive photoprotein targeted to the thylakoid lumen (TL) and thylakoid membrane (TM). For RT-PCR analyses, actin was used as housekeeping gene. For immunoblot analyses, total protein extracts (50 µg) were separated on 10% SDS-PAGE, transferred to PVDF and incubated with an anti-aequorin antibody (1: 1000 diluted). Double and single black arrows indicate the YFP-aequorin chimeras targeted to the thylakoid lumen and thylakoid membrane, respectively. White arrow indicates the aequorin chimera targeted to the cytosol, excluding the nucleus, in the *Arabidopsis* line $CPK17_{G2A}$-NES-YA, used here as a positive control.

Subsequent analysis of aequorin activity were carried out on the T2 generation, by screening 5 independent TL and TM sub-lines for aequorin-based total luminescence. After *in vivo* discharge, that gave positive results for all sublines (with luminescence levels ranging from 10^5 to $2 \cdot 10^6$ counts per seedling), one sub-line for every sub-chloroplast localization, characterized by the highest aequorin activity, was chosen to set up photoautotrophic cell suspension cultures.

Set up of photoautotrophic cell suspension cultures from *Arabidopsis* transgenic lines

To more easily check for the correct targeting of the YFP-aequorin chimeras and subsequently validate the system for *in vivo* Ca^{2+} measurements, autotrophic cell suspension cultures, containing chloroplasts as functional types of plastids, were set up. To this aim, *Arabidopsis* seeds were sown on hormone-enriched solid agar germination

medium (MS medium containing 3% sucrose, 0.5 mg/l 2,4-D and 0.25 mg/l 6-BaP) supplemented with 50 µM Basta. After about 3 weeks, thanks to the interaction between endogenous and exogenous hormones, a green mass of cells formed at the level of the hypocotyls (Fig. S1a and Fig. S1b). Those showing a higher YFP fluorescence were axenically cut and transferred into a sucrose-impoverished liquid medium (MS medium containing 2% sucrose, 0.5 mg/l 2,4-D and 0.25 mg/l 6-BaP) under relatively high light conditions (110 µmol photons $m^{-2}s^{-1}$, 16/8h light/dark cycle) to obtained autotrophic cell suspension cultures (Fig. S1c).

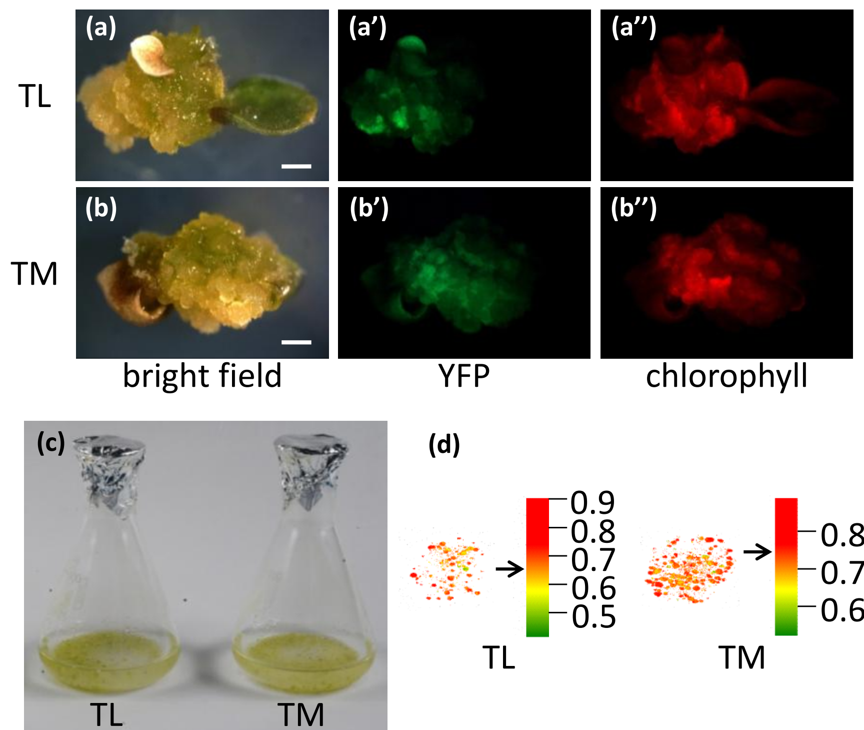

Fig. S1 Set up of *Arabidopsis* autotrophic cell suspension cultures from transgenic lines stably expressing YFP-aequorin in the thylakoid lumen (TL) and thylakoid membrane (TM), respectively. (a-a'' and b-b'') Observations at the fluorescence stereomicroscope of green calli derived from dedifferentiation of hypocotyls from *Arabidopsis* seeds germinated on hormone-enriched MS solid medium. (c) Photoautotrophic cell suspension cultures obtained after the transfer of green calli from solid to liquid medium containing a progressively reduced sucrose concentration (from 2% to 0.5%) under high light conditions (110 µmol photons $m^{-2}s^{-1}$). (d) PAM imaging analyses of *Arabidopsis* autotrophic cell suspension cultures derived from the TL (F_V/F_M value = 0.67) and TM (F_V/F_M = 0.72) lines.

Pulse amplitude modulation (PAM) analysis, that gives chlorophyll fluorescence values as output related to the efficiency of photosystem II, provided evidence about the condition of photosynthetic activity in the cell cultures. Both autotrophic cell suspension cultures displayed a high photosynthetic efficiency, as demonstrated by a F_v/F_m value of 0.67 and 0.72 for the TL and TM lines, respectively (Fig. S1d). Autotrophic cell suspension cultures were next used to verify the correct targeting of the fluorescently-

tagged Ca^{2+} reporter by means of confocal microscopy analyses and to perform Ca^{2+} measurements assays in different chloroplast sub-compartments.

Analyses of the subcellular localization of YFP-aequorin fusion proteins

The constructs generated in this work were designed to target the YFP-aequorin fusion proteins into the thylakoid lumen (TL line) and to the thylakoid membrane, exposed to the stroma (TM line). Confocal microscopy observations on suspension-cultured cells derived from the TL and TM lines showed that the fluorescent signal of YFP perfectly overlapped with the red chlorophyll fluorescence, suggesting that the recombinant proteins indeed reside inside chloroplasts (Fig. 3).

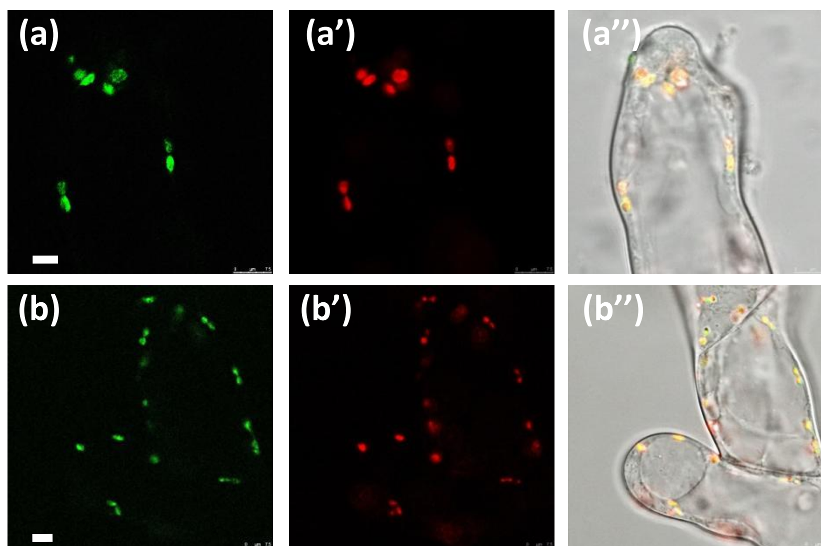

Fig. 3 Confocal microscopy analyses of YFP-aequorin subcellular localization in *Arabidopsis* suspension-cultured cells stably expressing the fusion proteins in the thylakoid lumen (a-a'') and thylakoid membrane (b-b''). Suspension-cultured cells were observed with a YFP filter (a-b) and a chlorophyll filter (a'-b'). In c-c' fluorescence microscopy images were merged with bright field images. Bar, 20 μm.

However, laser scanning confocal microscopy analyses did not allow to verify a precise localization of the recombinant proteins at the thylakoid system. As a consequence, immunocytochemical analyses were additionally performed on leaf fragments of the two *Arabidopsis* transgenic lines. Observations at the transmission electron microscopy (TEM) after immunogold labelling with the anti-aequorin antibody showed the presence of evident electron-dense gold particles at the level of the thylakoid system in both the TL (Fig. 4a) and TM lines (Fig. 4b). A wild-type line was used as negative control, confirming the lack of any gold-labelling, either in the cytosol or organelles, in particular chloroplasts (Fig. 4c). An *Arabidopsis* line stably expressing aequorin in the chloroplast stroma (Mehlmer et al., 2012) was used as a positive control, exhibiting evident gold particles in the most

electron-dense region of the chloroplast, corresponding to the stroma, among grana stacks and close to starch granules (Fig. 4d). Although immunogold labelling followed by TEM analyses provided good evidence for the expected localization of the newly-engineered aequorin chimeras directed at the thylakoid system, it was not possible to definitively ascertain their localization at the thylakoid lumen and thylakoid membrane, respectively.

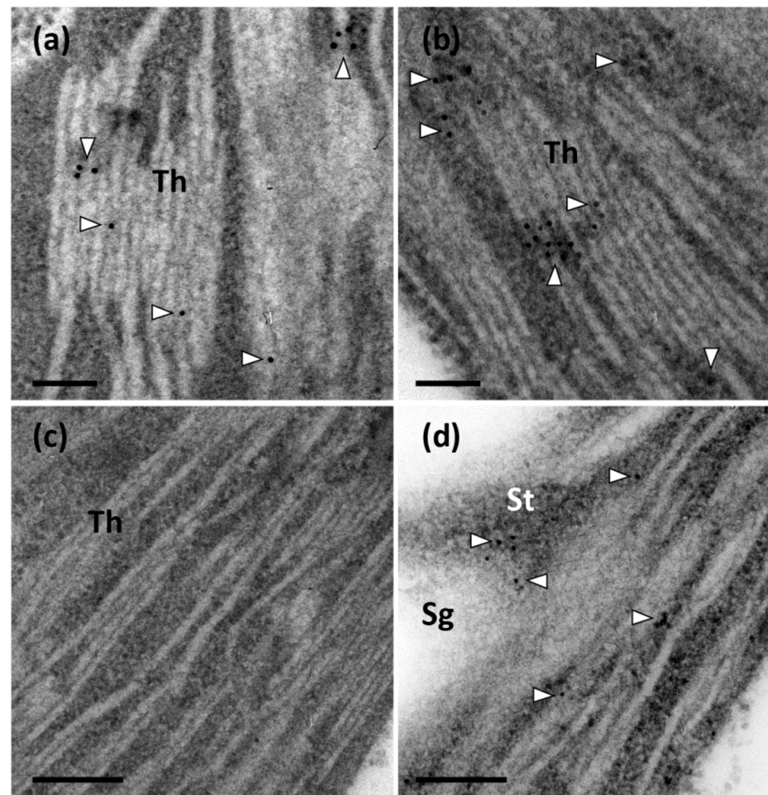

Fig. 4 Immunocytochemical analyses of YFP-aequorin subcellular localization in *Arabidopsis* transgenic lines stably expressing the fusion proteins in the thylakoid lumen (a) and thylakoid membrane (b). An *Arabidopsis* wild-type line (c) and a line expressing YFP-aequorin in the stroma (d) were used as negative and positive controls, respectively. White arrows indicate gold particles. Bar, a-b: 100 nm; c-d: 200 nm. Th, Thylakoids; St, Stroma; Sg, Starch granule.

To further support the correct subcellular localization of the thylakoid-targeted aequorin chimeras, a biochemical approach was also applied. Intact thylakoids were isolated from *Arabidopsis* rosette leaves of the TL and TM transgenic lines and incubated for 20 min with the proteolytic enzyme thermolysin. This treatment was expected to remove proteins that extrude from the thylakoid membrane, whereas lumenal proteins should be protected by thylakoid membrane. Immunoblot analyses showed that, as expected, aequorin targeted to the thylakoid lumen was not affected by the proteolytic treatment because protected inside the lumen, whereas aequorin targeted to the thylakoid membrane was totally removed, confirming its expected exposure towards the stroma of chloroplasts (Fig. 5).

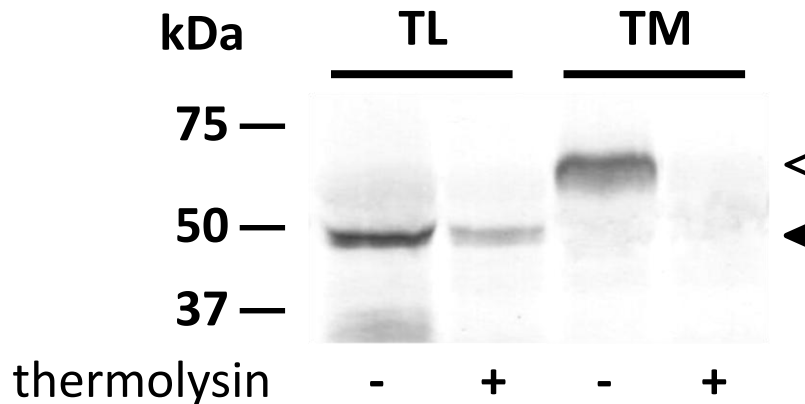

Fig. 5 Immunoblot analyses of isolated thylakoids from leaves of *Arabidopsis* transgenic lines stably expressing YFP-aequorin in the thylakoid lumen (TL) and thylakoid membrane (TM). Protein samples were incubated for 20 min in the absence (-) or presence of the proteolytic enzyme thermolysin (0.1 μg/μl), as indicated. Proteins (50 μg) were separated by 10% SDS-PAGE, transferred on PVDF and incubated with an anti-aequorin antibody (1:1000 diluted). Black and white arrow indicate the YFP-aequorin chimeras targeted to the thylakoid lumen and thylakoid membrane, respectively.

Taken together, these data unequivocally confirmed the correct subcellular localization of the thylakoid lumen- and thylakoid membrane-targeted aequorin probes.

Different environmental stimuli induced specific Ca^{2+} signals in the thylakoid lumen

Once the correct sub-chloroplast localization of the aequorin chimeras was definitely ascertained, the *Arabidopsis* transgenic lines were assayed in Ca^{2+} measurement experiments, to confirm their suitability to the monitoring of free [Ca^{2+}] changes in the thylakoid system. To this aim, autotrophic *Arabidopsis* cell suspension cultures stably expressing aequorin in the thylakoid lumen and thylakoid membrane were challenged with different physiological stimuli whose signal transduction is known to be mediated by Ca^{2+}. An *Arabidopsis* line stably expressing aequorin in the stroma (Mehlmer et al., 2012) was also used, to investigate if Ca^{2+} dynamics in the different subcompartments of chloroplasts are in some way connected. The rationale underlying the use of autotrophic cell cultures instead of seedlings *in toto* was the detection of only Ca^{2+} signals produced by chloroplasts, avoiding those possibly generated in the stroma of non-green plastids, for example in roots.

In resting conditions (in the dark), [Ca^{2+}] in the thylakoid lumen was estimated to be kept at relatively high concentration, *i.e.* about 0.5 μM (Fig. 6a). This concentration appears to be 3-to-5 fold higher than Ca^{2+} level (100÷150 nM) in the bulk stroma and in the stromal region just outside the thylakoid membrane (Fig. 6a). Treatment of suspension-

cultured cells with 10 mM H_2O_2 and 0.3 M NaCl were used to mimic two abiotic stimuli (oxidative stress and salt stress, respectively), whereas 20 µg/ml oligogalacturonides with a degree of polymerization of 9 to 18, pectic fragments of the plant cell wall, were applied to simulate a pathogen attack. The perception of all considered stimuli triggered specific changes in [Ca^{2+}] in all chloroplast subcompartments, except oligogalacturonides that failed to cause any detectable Ca^{2+} rise in the thylakoid lumen (Fig. 6b-d).

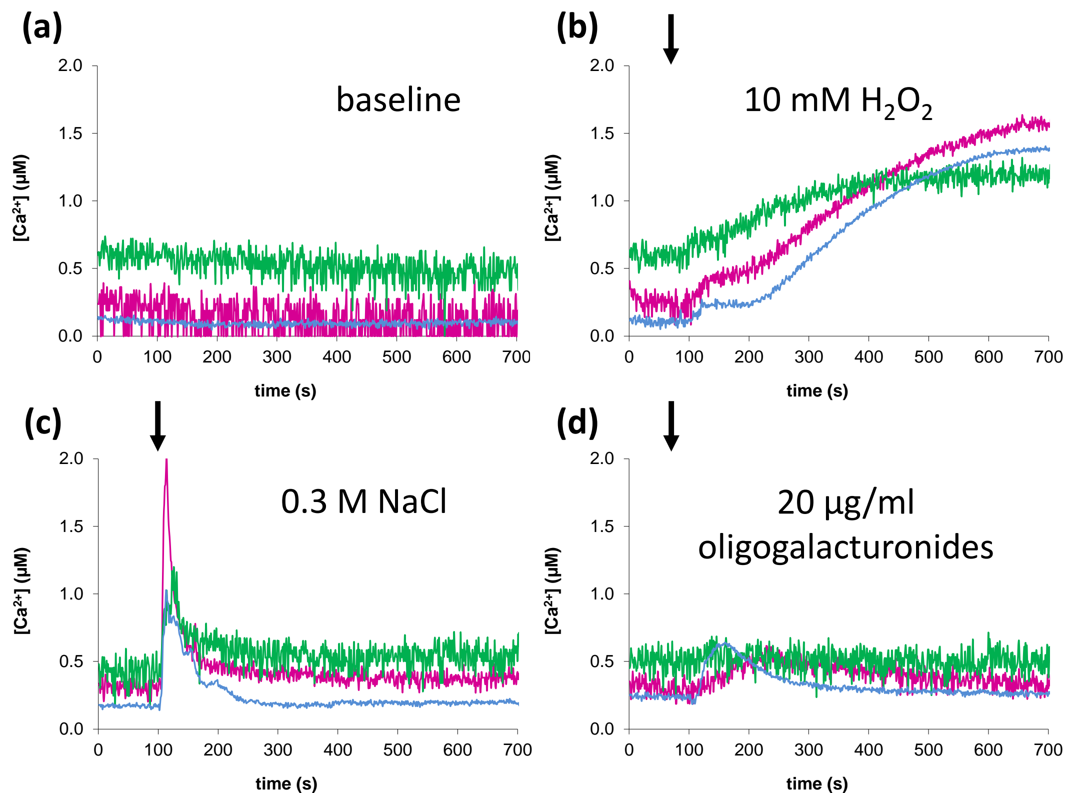

Fig. 6 Monitoring of sub-chloroplast free [Ca^{2+}] in response to environmental stimuli in *Arabidopsis* autotrophic cell suspension cultures stably expressing aequorin in the stroma (blue trace), thylakoid membrane (pink trace) and thylakoid lumen (green trace). Ca^{2+} measurements were carried out in resting conditions (a), or after challenge with different stimuli, as indicated in each panel. Black arrows indicate the timing of injection of the different stimuli (after 100 s). These and the following traces are representatives of at least three independent experiments which gave similar results.

Oxidative stress triggered a Ca^{2+} increase at the level of the external surface of the thylakoid membrane that closely mirrored the Ca^{2+} trace recorded in the bulk stroma. Ca^{2+} dynamics in the thylakoid lumen followed the trend recorded in the other two chloroplast locations, but starting from a higher level (Fig. 6b). Concerning salt stress, 0.3 M NaCl triggered very rapid and transient [Ca^{2+}] elevations in all three chloroplast sub-compartments. Interestingly, an evident microdomain of high [Ca^{2+}] was found to be transiently established in proximity of the thylakoid membrane, whose dissipation seems to

temporally coincide with the Ca^{2+} increase in the thylakoid lumen (Fig. 6c). These data suggest that the thylakoid lumen may be involved in switching off stromal Ca^{2+} signals.

Oligogalacturonides induced a transient Ca^{2+} change in the bulk stroma, and a similar Ca^{2+} variation also in the stromal region just outside the thylakoid membrane, although characterized by a slower dynamic. Interestingly, no detectable Ca^{2+} changes were triggered in the thylakoid lumen (Fig. 6d). In view of the high basal [Ca^{2+}] in the thylakoid lumen, and the moderate Ca^{2+} increase recorded in the stroma in response to oligogalacturonides, it is possible that the transient [Ca^{2+}] rise in the latter compartment in response to oligogalacturonides was not sufficient to induce a [Ca^{2+}] elevation in the thylakoid lumen, mediated by potential passive Ca^{2+} transporters located at the thylakoid membrane.

Taken together, these results confirm the functionality of the two aequorin chimeras targeted to the thylakoid lumen and thylakoid membrane, respectively, to detect Ca^{2+} changes in these chloroplast sub-locations and demonstrate the involvement of the thylakoid system in intracellular Ca^{2+}-mediated perception of environmental stimuli.

Transition from light to dark triggered intra-chloroplast Ca^{2+} fluxes without the involvement of cytosolic Ca^{2+} transients

As light is one of the most important factors for plant growth and development, the effect of a lights-off stimulus on chloroplast Ca^{2+} dynamics was considered. Ca^{2+} measurement assays in response to light-to-dark transition were carried out in *Arabidopsis* transgenic lines stably expressing aequorin in different subcellular locations, *i.e.* in the cytosol, cytosolic surface of the outer membrane of the plastid envelope, chloroplast stroma, in the thylakoid membrane and thylakoid lumen. In this set of experiments entire seedlings, instead of plant cell suspension cultures, were used. Indeed, in some cases challenge of the organism *in toto* with defined stimuli represent a condition that may more closely mimic the actual *in vivo* situation, especially when a systemic integration may be necessary to obtain an overall Ca^{2+}-mediated response.

In Fig. 7 the dark-induced [Ca^{2+}] changes occurring in the different subcellular locations are shown. Notably, variations in [Ca^{2+}] were recorded in different intra-chloroplast locations (Fig. 7c-e), but neither in the bulk cytosol (Fig. 7a) nor at the cytosolic surface of the plastid envelope (Fig. 7b). These data conclusively demonstrate, as previously suggested (Nomura et al., 2012) that the cytosol is not involved in [Ca^{2+}] changes in response to light-to-dark transition.

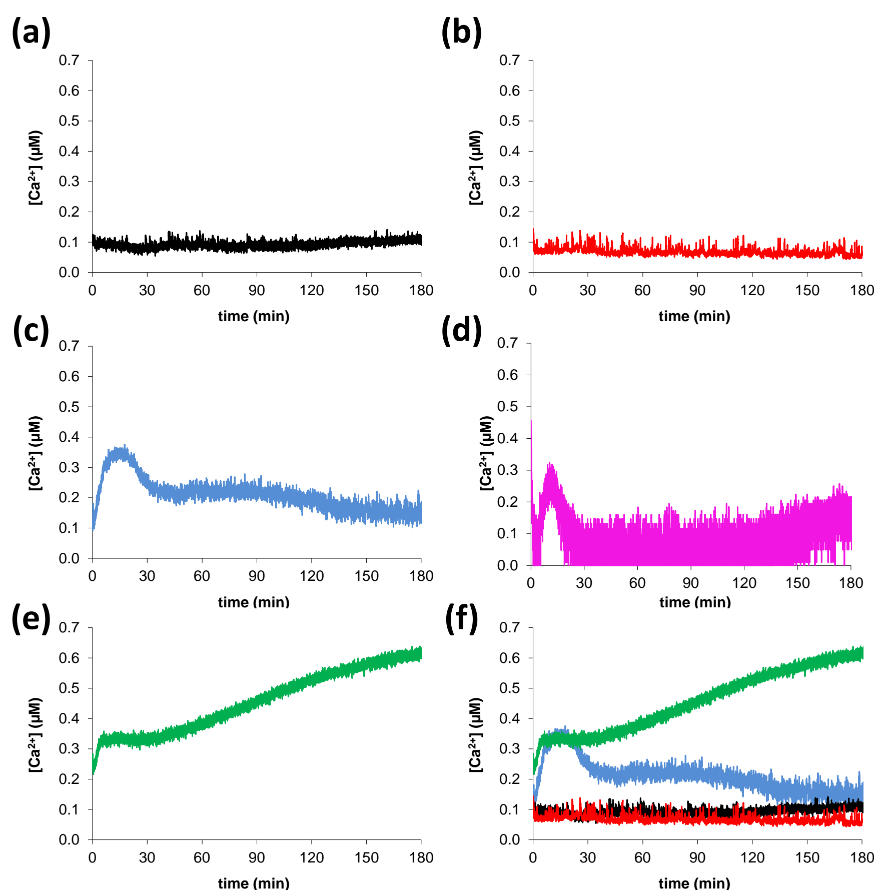

Fig. 7 Monitoring of Ca^{2+} fluxes in response to light-to-dark transition in *Arabidopsis* seedlings stably expressing YFP-aequorin chimeras in different subcellular locations, *i.e.* cytosol (a, black trace), external surface of the plastid outer envelope (b, red trace), stroma (c, blue trace), thylakoid membrane (d, purple trace) and thylakoid lumen (e, green trace). In f, Ca^{2+} traces recorded in the different transgenic lines were overlapped. In all graphs the lights-off stimulus starts at time 0.

In agreement with previous reports (Sai & Johnson, 2002; Nomura et al., 2012) the lights-off stimulus was found to trigger a transient [Ca^{2+}] increase in the stroma, that peaked at about 0.35 µM after 20 min and slowly decayed to basal level within 3 h (Fig. 7c). A similar Ca^{2+} dynamic, but characterized by a narrower peak, was observed in the stromal microdomain just outside the thylakoid membrane (Fig. 7d).

Interestingly, the dynamics of the Ca^{2+} trace recorded in the thylakoid lumen was found to be very different. In particular, [Ca^{2+}] was found to increase from a basal level of about 0.2 µM to about 0.35 µM within 10 min, followed by an intermediate [Ca^{2+}] plateau between 10 and 30 min. [Ca^{2+}] then continued to gradually increase, reaching a value of about 0.6 µM after 3 h (Fig. 7e).

The overlap of the Ca^{2+} traces recorded in the different subcellular locations (Fig. 7f) indicates that: i) the dark-induced intra-chloroplast Ca^{2+} fluxes originate from inside the organelle, in view of the lack of any detectable Ca^{2+} changes in either the cytosol or

chloroplast surface; ii) the intermediate [Ca^{2+}] plateau observed in the thylakoid lumen temporally coincides with the transient Ca^{2+} rise in the stroma, suggesting a potential involvement of the thylakoid system as Ca^{2+} reservoir that can be mobilized upon light-to-dark transition.

An additional experiment, carried out in seedlings that had been exposed to 6 h of light and then rapidly moved to darkness in the luminometer chamber, confirmed that [Ca^{2+}] is indeed kept at 0.2 μM in the thylakoid lumen during the light phase, and that the lights-off stimulus induces a gradual increase in thylakoid [Ca^{2+}] (Fig. S2a). Moreover, at the end of the darkness phase, *i.e.* between the 8th hour of dark and the 1st hour of light in the new day, [Ca^{2+}] was found to be maintained at high levels (0.5÷0.6 μM), and this concentration is kept unchanged in a condition of absence of light during the beginning of the new day (Fig. S2b). The high level of Ca^{2+} recorded in the thylakoid lumen at the end of light-to-dark experiments (Fig. 7e and Fig. S2b) perfectly matched the thylakoid [Ca^{2+}] monitored in resting conditions in autotrophic cell suspension cultures (Fig. 6a).

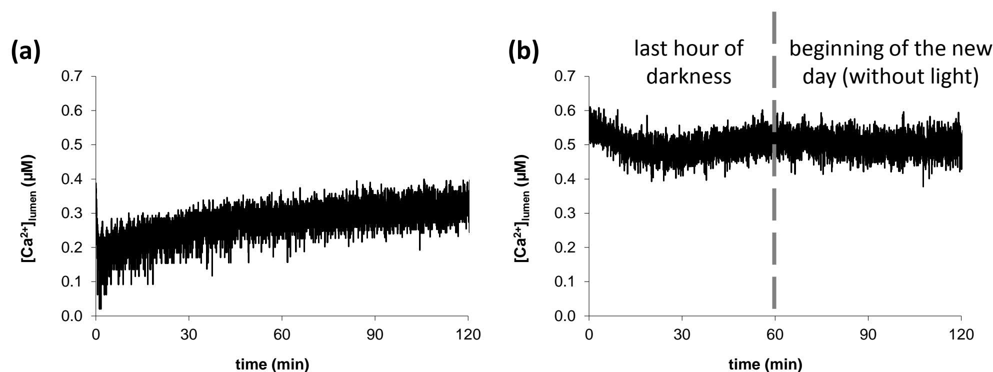

Fig. S2 Monitoring of thylakoid lumenal [Ca^{2+}] in seedlings that were transferred to the luminometer chamber (light-to-dark transition) (a) after 6 h of light or (b) at the end of the dark phase (8 h).

To shed some light on the origin of dark-induced intra-chloroplast Ca^{2+} fluxes, a pharmacological approach was applied. *Arabidopsis* seedlings were pre-treated for 30 min before the light-to-dark transition with 5 μM nigericin, a ionophore that acts as a K^+ ionophore promoting K^+/H^+ exchange across membranes and disrupts ΔpH across thylakoid membranes without affecting the electrical gradient. As shown in Fig. 8a, the disruption of the H^+ gradient across the thylakoid membrane inhibited the dark-induced Ca^{2+} uptake into the thylakoid lumen. This result is in agreement with the postulated presence of a Ca^{2+}/H^+ antiport in the plant chloroplast thylakoid membrane (Ettinger et al., 1999). It has to be noted, however, that in the presence of nigericin a rapid and transient Ca^{2+} rise was recorded, that peaked at about 1 μM after a few min and then was promptly

dissipated within 30 min. The rapid activation of this Ca^{2+} flux leads to hypothesize the involvement of a putative Ca^{2+}-permeable channel localized at the thylakoid membrane (Sello et al., unpublished results; see Chapter 6). Indeed, the inhibition of the Ca^{2+}/H^+ exchanger and the resulting accumulation of Ca^{2+} in the stroma may be responsible for the activation of passive mobilization pathways for Ca^{2+} at thylakoid membranes.

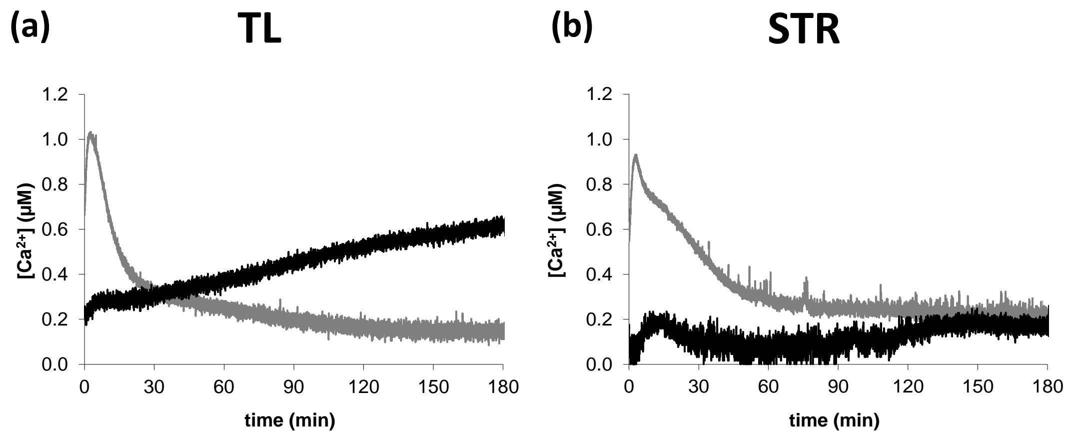

Fig. 8 Effect of pre-treatment with nigericin on dark-induced Ca^{2+} dynamics in the thylakoid lumen and stroma. *Arabidopsis* seedlings stably expressing aequorin in the thylakoid lumen (a) or stroma (b) were subjected to light-to-dark transition in control conditions (black traces) or after 30 min pre-treatment with 5 µM nigericin (grey trace).

Concerning the chloroplast stroma, the transient [Ca^{2+}] increase activated in response to dark displayed a higher amplitude in the presence of nigericin, and maintained a higher steady-state after 30 min from the light-to-dark transition, without decreasing to the basal levels observed in control conditions (Fig. 8b). This result support the notion that the dark-induced Ca^{2+} rise may be amplified as a consequence of the inhibition of Ca^{2+} uptake in the thylakoid lumen caused by nigericin. Taken together, these data suggest a complex scenario in which the thylakoid system may act as a rapidly mobilizable Ca^{2+} store, responsible for both the origin and dissipation of stromal Ca^{2+} signals.

Discussion

A fine-tuned regulation of intracellular [Ca^{2+}] is fundamental for a proper cellular homeostasis and functioning of Ca^{2+}-based signal transduction pathways in all eukaryotic cells. In plant cells, most of the research in this field has been focused so far on the generation of transient cytosolic Ca^{2+} changes, as well as on the molecular components involved in decoding cytoplasmic Ca^{2+} signals, characterized by stimulus-specific spatio-

temporal parameters. However, knowledge about the precise involvement of the different intracellular compartments of the plant cell in Ca^{2+} homeostasis and signalling far lags behind, in comparison with the large body of information currently available in the animal field, where the contribution of *e.g.* the endo/sarcoplasmic reticulum and mitochondria has been clearly established (Berridge et al., 2000; Clapham, 2007; Brini et al., 2013). However, it is increasingly becoming apparent that Ca^{2+} fluxes across organellar membranes not only can shape cytosolic Ca^{2+} signatures, but also regulate metabolic processes inside organelles (Stael et al., 2012; Nomura & Shiina, 2014). Concerning chloroplasts, unique organelles of the plant cell where the light-powered reactions of photosynthesis take place, the relationship between these organelles and calcium is still elusive (Rocha & Vothknecht, 2012). Ca^{2+} is known to cover essential roles in the physiology of the organelle, both in the regulation of photosynthesis (*e.g.* the assembly and proper functioning of photosystem II, the regulation of stromal enzymes for CO_2 fixation) and also other processes (*e.g.* the import of nuclear-encoded proteins, the plastid division) (Rocha & Vothknecht, 2012; Hochmal et al., 2015). Nevertheless, little information is so far available about the participation of chloroplasts in the plant Ca^{2+} signalling network, *e.g.* in the transduction of environmental signals. To elucidate the Ca^{2+} handling ability of chloroplasts, it is essential to monitor in a sensitive and accurate way the concentration of the ion and its variations in the different organellar sub-compartments. Recently, a novel set of YFP-fused aequorin chimeras targeted to the outer and inner surface of the chloroplast envelope membranes and stroma have been generated (Mehlmer et al., 2012). In this work, two additional variants of aequorin, targeted to the thylakoid lumen and thylakoid membrane (exposed to the stroma) were designed and *Arabidopsis* plant and cell lines stably transformed with the above constructs were generated for *in vivo* Ca^{2+} measurements. Aequorin targeting to the two sub-chloroplast localizations was obtained by fusing the coding sequence of YFP-aequorin with nucleotide sequences coding for proteins localized in the considered sub-compartments. Among the many advantages of aequorin as a Ca^{2+} reporter, there is its ability to function over a wide range of $[Ca^{2+}]$ and its nearly insensitiveness to changes in pH (Brini, 2008), which renders this genetically encoded Ca^{2+} indicator ideal to reliably monitor $[Ca^{2+}]$ in the chloroplast thylakoid system. Once the correct localization of the two aequorin chimeras was confirmed by using a combination of laser scanning confocal microscopy, immunogold labelling and biochemical analyses, Ca^{2+} measurement assays in response to various environmental stimuli were carried out. Monitoring of $[Ca^{2+}]$ in transgenic *Arabidopsis* cell lines confirmed their suitability to

monitor [Ca^{2+}] variations just outside the thylakoids and within the thylakoid lumen in response to environmental stimuli. In resting conditions Ca^{2+} was found to be maintained in the thylakoid lumen at a concentration (~ 0.5 μM) which is 3÷5 fold higher than in the chloroplast stroma (0.1-0.15 μM). Interestingly, different environmental cues, such as oxidative and salt stresses, were found to trigger chloroplast Ca^{2+} signals not only in the chloroplast stroma but also inside the thylakoid lumen, characterized by specific kinetic parameters.

In view of the importance of the light signal for the physiology of plants, the effect of light-to-dark transition on intracellular Ca^{2+} levels was also evaluated. It has been demonstrated that the lights off stimulus induces a slow long-lasting [Ca^{2+}] increase in the stroma of chloroplasts (Sai & Johnson, 2002; Nomura et al., 2012). To investigate if the darkness may induce particular Ca^{2+} dynamics at the level of the thylakoid system, changes in [Ca^{2+}] in the *Arabidopsis* lines stably expressing aequorin in the thylakoid membrane and in the thylakoid lumen were monitored in response to light-to-dark transition. Three additional transgenic plant lines, expressing aequorin in the cytosol (excluding the nucleus), the outer surface of chloroplasts and the stroma (Mehlmer et al., 2012) were also used, to elucidate whether Ca^{2+} fluxes solely exist between the thylakoid lumen and the stroma or whether in and out fluxes from and to the cytosol may also play a role in this process. The lack of any detectable Ca^{2+} rise in either the bulk cytosol or cytosolic microdomain close to the chloroplast envelope (Fig. 7a-b), demonstrated that the cytosol is not involved in the stromal [Ca^{2+}] change in response to light-to-dark transition, as recently suggested (Nomura et al., 2012). Moreover, Ca^{2+} measurement assays in the thylakoid lumen revealed unexpected Ca^{2+} dynamics in this chloroplast sub-compartment in response to variations in light conditions. In particular, we found that the light-to-dark transition induced an unexpected long-lasting and sustained [Ca^{2+}] increase in the thylakoid lumen: indeed, [Ca^{2+}] was assessed to be at a relatively low level (0.2 μM) at the end of the day (*i.e.* after 16 h of light), whereas the lights-off stimulus was found to induce a gradual increase in thylakoid lumenal [Ca^{2+}], reaching 0.5÷0.6 μM within 3 h after the start of darkness (Fig. 7e). This high [Ca^{2+}] is subsequently kept unchanged during the whole night (Fig. S2). A comparison between the Ca^{2+} traces recorded in the different sub-chloroplast locations demonstrated that the intermediate plateau in [Ca^{2+}] reached in the thylakoid lumen exactly matched with the timing of the transient stromal Ca^{2+} increase (Fig. 7f). This suggests that the thylakoid lumen might serve as a Ca^{2+}-releasable store for the Ca^{2+} fluxes observed in the stroma, as it had previously been hypothesized (Sai & Johnson, 2002). On

the other hand, the possibility that Ca^{2+} may be released from Ca^{2+} binding proteins located in the thylakoid membrane, such as CAS (Nomura et al., 2008) cannot be ruled out. Our recent finding that the transient stromal Ca^{2+} signal in response to light-to-dark transition occurred only in chloroplasts, but not in non-green plastids of *A. thaliana* cell cultures (Sello et al., 2015, accepted paper; Chapter 3) further strengthens the notion that the light off-induced Ca^{2+} fluxes may indeed derive from the thylakoid system.

To try to unravel the origin of these intra-chloroplast Ca^{2+} fluxes, a pharmacological approach using nigericin, a ionophor catalyzing the exchange of H^+/K^+, thus dissipating the thylakoid proton gradient without affecting the electrical gradient, was adopted. The results indicated the likely involvement of a Ca^{2+}/H^+ antiporter, previously hypothesized to be localized at the thylakoid membranes (Ettinger et al., 1999), in the dark-induced Ca^{2+} uptake into the thylakoid lumen. Ca^{2+} measurement assays carried out in the presence of nigericin, however, also showed the unexpected appearance of a fast Ca^{2+} increase in the thylakoid lumen, that was equally quickly dissipated. It is likely that the effective inhibition of the Ca^{2+}/H^+ exchanger activity might have caused a transient microdomain of high [Ca^{2+}] just outside the thylakoid membrane, leading to the opening of an as yet unidentified Ca^{2+}-permeable channel potentially located at the thylakoid membrane. Further investigations are needed to unravel the identity of putative Ca^{2+}-permeable channels localized at plastid membranes in the organellar Ca^{2+} fluxes evoked by different environmental stimuli (see Chapter 6).

It would also be interesting to know whether the corresponding transition from dark to light (at the beginning of the day) triggers a decay in the thylakoid lumenal [Ca^{2+}] characterized by a temporal kinetics similar to that observed upon the lights-off stimulus. Unfortunately the Ca^{2+}-sensitive photoprotein aequorin is not suitable for Ca^{2+} measurements under light conditions, analogously to GFP-based Ca^{2+} probes such as cameleon, although for different reasons.

In conclusion in this work, thanks to the successful targeting of the genetically encoded Ca^{2+} indicator aequorin to the thylakoid system, precise estimates of the thylakoid luminal Ca^{2+} content and changes therein in response to several environmental cues were given for the first time. The obtained results indicate that the thylakoid system is involved in the modulation of chloroplast Ca^{2+} signatures, by either switching off (*e.g.* after a salt stress) or originating (*e.g.* after light-to-dark transition) stromal Ca^{2+} signals. The former event might be essential to restore low basal stromal [Ca^{2+}] after a Ca^{2+} signalling event, whereas the latter has been hypothesized to regulate the activity of some enzymes of the

Calvin- Benson cycle, resulting in an inhibition of CO_2 fixation during the night (Johnson et al., 2006; Hochmal et al., 2015). It remains to be established whether thylakoid Ca^{2+} signals may also play some role in the regulation of specific processes occurring in the thylakoid lumen. These novel aspects of chloroplast Ca^{2+} dynamics updates the role of this organelle and its sub-compartments in the Ca^{2+} regulation network of the plant cell.

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CHAPTER 6.

Involvement of a plastid-localized homologue of the mammalian mitochondrial calcium uniporter (MCU) in plastidial Ca^{2+} fluxes triggered by environmental stimuli in *Arabidopsis thaliana*

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Abstract

Calcium (Ca^{2+}) is involved in the regulation of several processes in chloroplasts, including photosynthesis, the import of nuclear-encoded proteins and signalling. Despite the importance of organellar Ca^{2+} fluxes for the control of organellar functions, only very little is known about the Ca^{2+} transport mechanisms operating at plastidial membranes. To investigate the involvement of a putative plastidial homologue of the mammalian mitochondrial calcium uniporter (MCU) in the organellar Ca^{2+} fluxes evoked by different environmental stimuli, an *Arabidopsis thaliana* mutant knock-out line defective in this Ca^{2+}-permeable transporter was stably transformed with a construct encoding stroma-targeted aequorin. Ca^{2+} measurement assays demonstrated significant differences in the Ca^{2+} dynamics recorded in the knock-out *versus* the wild-type line, suggesting a potential participation of the plastidial MCU homolog in the dissipation of stromal Ca^{2+} signals. On the basis of the obtained data, a potential localization of the chloroplast MCU homologue at thylakoid membranes is proposed.

Work in progress, to be included, when finished, in a wider manuscript

Introduction

Calcium (Ca^{2+}) is used by all organisms to transduce the information derived from external/internal stimuli. In resting conditions, the cytosolic concentration of the ion ($[Ca^{2+}]_{cyt}$) has to be maintained at levels much lower than the extra-cytoplasmic compartments (about 10000-fold difference) in order not to interfere with the phosphate metabolism; for this reason, Ca^{2+} must be compartmentalized (Plattner & Verkhratsky, 2015).

Plants, as sessile organisms, have evolved specific mechanisms to respond and adapt to changes in environmental conditions. Intracellular transduction of information mediated by Ca^{2+} is well established to be the most relevant in these organisms as many elements involved in Ca^{2+} handling have been found to take part in Ca^{2+} dynamics. Ca^{2+} signals are specific for distinct stimuli, and they are characterized by peculiar variations in $[Ca^{2+}]$ in time and in space (McAinsh & Pittman, 2005; Dodd et al., 2010). This information is then decoded downstream by Ca^{2+}-binding proteins acting as Ca^{2+} sensors that lead to proper physiological responses, making plants able to cope with the changes in external conditions (Hashimoto & Kudla, 2011). Although there is reasonable knowledge about the transport machinery located at the plasma membrane and tonoplast, allowing to maintain a proper $[Ca^{2+}]_{cyt}$ compatible with life and permitting at the same time the occurrence of stimulus-specific cytosolic Ca^{2+} signals (Dodd et al., 2010), the identity of the molecular components present in other subcellular compartments, such as chloroplasts, are still poorly understood. Although putative members responsible for chloroplast Ca^{2+} fluxes have been proposed (Stael et al., 2012; Nomura & Shiina, 2014), the key actors as well as the regulatory mechanisms are far from being clarified. Information regarding the Ca^{2+} transport systems in this organelle are limited to biochemical evidence for a Ca^{2+}/H^+ antiporter at the thylakoid membrane (Ettinger et al., 1999) and the characterization of two glutamate receptors (AtGLR3.4 and AtGLR3.5) localized in the plastid envelope (Teardo et al., 2010, 2011, 2015).

In this work, our attention has been focused on a membrane protein putatively involved in plastidial Ca^{2+} fluxes. The gene coding for this putative 37 kDa protein has been identified in the *Arabidopsis thaliana* genome by bioinformatic analyses, since it includes a chloroplast targeting peptide and shows a sequence similarity of about 35% with the recently identified mammalian mitochondrial calcium uniporter (MCU) (De Stefani et al., 2011). Evidence supporting the presence of such a component includes measurement of

Ca^{2+} transport across the inner envelope membrane of pea chloroplasts possibly through a Ca^{2+} uniport-related mechanism (Roh et al., 1998).

In the *Arabidopsis thaliana* genome six genes are present that shares a rather low homology to the human MCU: the encoded proteins in particular share the pore-forming region, made of two transmembrane helices connected by a highly conserved loop domain containing the amino acid sequence DVME (DIME in human) (Stael et al., 2012). According to bioinformatic predictions, all these potential *Arabidopsis* MCU isoforms localize to mitochondria, except for At5g66650, which is equally predicted to target chloroplasts (Schwacke et al., 2003). The possibility that this MCU isoform has a chloroplast localization is supported by previous reports about a light-dependent Ca^{2+} influx pathway in chloroplasts, displaying properties similar to those observed for the mammalian MCU and inhibited by ruthenium red (Kreimer et al., 1985).

Here, we demonstrate that the chloroplast MCU homologue is involved in plastidial Ca^{2+} fluxes in response to environmental stimuli. Variations in $[Ca^{2+}]$ were monitored in wild-type and an MCU knock-out mutant stably expressing the Ca^{2+}-sensitive photoprotein aequorin in the stroma of *Arabidopsis* seedlings. Moreover, a pharmacological approach was also used, based on the MCU specific inhibitor NecroX-5 (Thu et al., 2012). On the basis of the observed Ca^{2+} dynamics, a novel intra-chloroplast localization for the plastidial MCU homologue is proposed. Further investigations are needed to ascertain the precise location and functions of this intriguing novel chloroplast Ca^{2+}-permeable transporter.

Materials and methods

Plant material

Arabidopsis thaliana ecotype Columbia (Col-0) plant lines stably expressing YFP-aequorin and targeted to the plastid stroma by fusion with the first 85 amino acids of the chloroplast NADPH-dependent thioredoxin reductase C (NTRC) (Mehlmer et al., 2012) were used in this study. Moreover, an *Arabidopsis thaliana* knock-out mutant line, defective for the mammalian MCU homologue localized in the chloroplast (*At5g66650*), was purchased from the European *Arabidopsis* Stock Centre, SK Collection (NASC, n° 1003356). This homozygous line is resistant to Basta.

Agrobacterium tumefaciens transformation

The chloroplast MCU knock-out line was transformed with either the construct encoding YFP-aequorin targeted to the plastid stroma (Mehlmer et al., 2012), or the construct encoding YFP-aequorin targeted to the thylakoid lumen (Sello et al., unpublished; see Chapter 5), both carrying kanamycin resistance. The transformation was performed with the floral dip method, using *Agrobacterium tumefaciens* strain GV3101 (Clough & Bent, 1998). Potential transformants of the T1 generation were selected by growth on half-strength MS plates containing 50 μg/ml kanamycin.

Aequorin expression analyses

Total RNA was extracted from leaves of 1 month-old kanamycin-resistant plants of the T1 generation using the "RNeasy Plant Mini Kit" (Qiagen) and reverse transcribed with SuperScript® III (Invitrogen) according to manufacturer's instructions. RT-PCR analysis of gene expression was performed using primers designed on the aequorin cDNA sequence (forward 5'-tcgacaacccaagatggattgga-3'; reverse 5'-tgatagcatgcgaattcatcagtgttttat-3'). The coding sequence of actin was used as housekeeping gene (forward: 5'-ggttgcaccgccagagagaaaatac-3'; reverse 5'-aacaaactcaccaccacgaaccaga-3').

Immunoblot analysis were carried out on total protein extracts from leaves of 1 month-old plants. Samples were separated by 10% SDS-PAGE and blotted on PVDF membrane (Immobilon). Afterwards, incubation with 1:10000 monoclonal anti-aequorin antibody (Abcam, Cambridge, UK) was performed, as previously described (Zonin et al., 2011).

Wild-type *Arabidopsis* plants were used as negative control in both types of analyses.

Confocal laser scanning microscopy

The localization of YFP-aequorin chimera in *Arabidopsis* leaf mesophyll cells of seedlings of the T1 generation was analyzed with the confocal microscope Leica TCS SP5 II (Leica Microsystems) mounted on a Leica DMI6000 inverted microscope with automated programmable scanning stage and motorized lens turret. Images were collected with the Leica Application Suite software (LAS AF, Leica Microsystems). Excitation with the Ar laser was carried out at 488 nm and the emitted fluorescence was detected at 505-530 nm for YFP and at 680-720 nm for chlorophyll.

Monitoring of stromal Ca^{2+} dynamics in response to environmental stimuli

Seeds of *Arabidopsis* wild-type and MCU knock-out plants stably expressing aequorin in the chloroplast stroma seeds were surface-sterilized and sown on half-strenght MS medium containing 1.5% sucrose and 50 μg/ml kanamycin to germinate under a 16 h/8 h light-dark cycle at 24°C. 7-to-14 day-old seedlings were reconstituted overnight with 5 μM coelenterazine. On the following day, seedlings were transferred in the luminometer chamber containing 300 μl H_2O and challenged with different stimuli, *i.e.* 0.3 M NaCl, 0.6M mannitol, cold shock (injection of 3 volumes of ice-cold H_2O) and 10 mM H_2O_2. $[Ca^{2+}]_{str}$ variations were monitored for 30 min and total aequorin was discharged at the end of every experiment by injection of 0.33 M $CaCl_2$ 10% ethanol. The light signal was collected and converted off-line into Ca^{2+} concentration values using a computer algorithm based on the Ca^{2+} response curve of aequorin (Brini et al., 1995). In some experiments, wild-type seedlings were pretreated for 30 min with 50 μM NecroX-5 (Santa Cruz Biotechnology).

Statistical analysis

Student's *t*-test was used to analyze the statistical significance of the difference observed between the Ca^{2+} peaks in the wild-type and MCU knock-out lines. Data are shown as means ± S.E. of at least five independent experiments.

Results

Generation of an *Arabidopsis* knock-out line defective in the plastidial MCU isoform and stably expressing aequorin in the stroma

An *Arabidopsis* knock-out mutant line defective for the mammalian MCU homologue localized in the chloroplast (*At5g66650*) (European *Arabidopsis* Stock Centre, SK Collection NASC, n° 1003356) was transformed with the construct encoding YFP-aequorin targeted to the stroma (Mehlmer et al., 2012). RT-PCR analyses of gene expression and immunoblot analyses on kanamycin-resistant plants (T1 generation) confirmed the expression of aequorin in two *Arabidopsis* sublines (#6 and #7) (Fig. 1a and 1b). Confocal microscopy analyses were also carried out, in order to confirm the correct localization of the YFP-aequorin probes to chloroplasts in the transgenic MCU knock-out

lines. A YFP signal that perfectly merged with chloroplasts dimension and shape, highlighted by chlorophyll autofluorescence, was clearly evident (Fig. 1c). The exact sub-chloroplast localization of this aequorin chimera to the organellar stroma has been assessed in previous work in transgenic *Arabidopsis* plants transformed with the NTRC-YA construct (Mehlmer et al., 2012; Sello et al., unpublished; see Chapter 5).

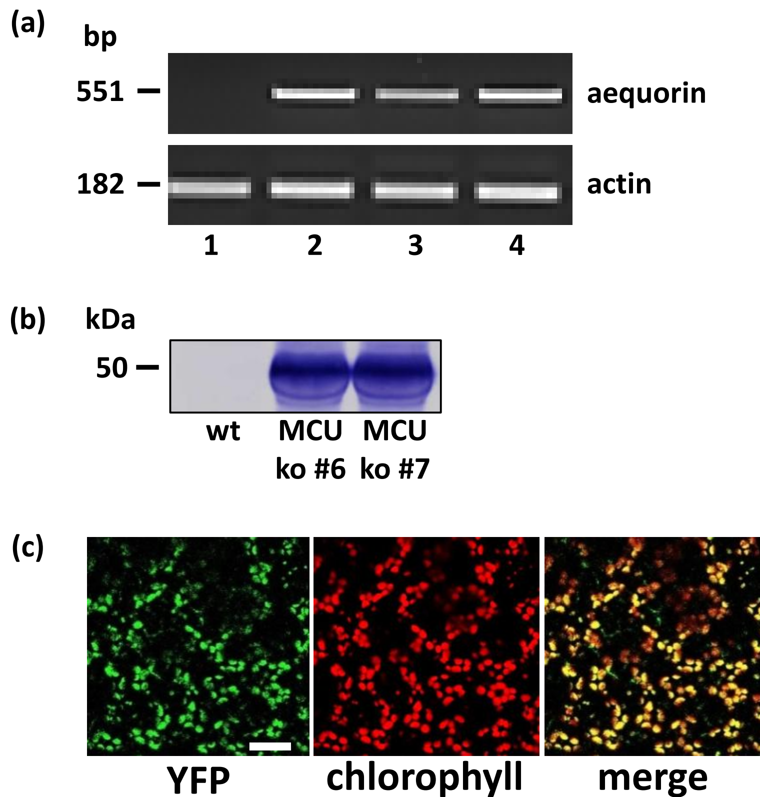


Fig. 1 Analyses of aequorin expression and intracellular localization in an *Arabidopsis* MCU knock-out line stably transformed with the construct encoding a YFP-aequorin chimera targeted to the plastid stroma. (a) RT-PCR gene expression analysis. A wild-type line (lane 1) and the line expressing aequorin in the chloroplast stroma (lane 2) were used as negative and positive control, respectively. Two positive transformants (MCU ko #6 and MCU ko #7) were identified (lane 3 and 4). Actin was used as housekeeping gene. (b) Immunoblot analysis. Total protein extracts (50 µg) were separated on 10% SDS-PAGE, transferred to PVDF and incubated with an anti-aequorin antibody (1: 1000 diluted). (c) Confocal microscopy analyses of leaf mesophyll cells from one of the two transgenic *Arabidopsis* sub-lines (MCU ko #6), shown here as representative. Bar, 20 µm.

The plastidial MCU homologue is involved in the organellar Ca^{2+} fluxes in response to environmental stimuli

The proposed chloroplast localization of this MCU homologue, together with its Ca^{2+}-transporting properties that have been demonstrated in parallel electrohysiological experiments by members of the Szabò's lab (Teardo et al., unpublished results), stimulated the analysis of its possible involvement in mediating chloroplast Ca^{2+} fluxes after the perception of environmental stimuli. To this aim, aequorin-based Ca^{2+} measurements

assays in response to abiotic stresses were carried out in the MCU knock-out stably expressing aequorin targeted to the stroma. Variations in stromal [Ca^{2+}] triggered by salinity, drought, cold and oxidative stress were monitored in entire seedlings and compared with the corresponding Ca^{2+} dynamics recorded in the wild-type transformed with the same construct. Transient [Ca^{2+}] elevations recorded in the stroma of the knock-out mutant are higher than in the wild-type in response to all the considered stimuli. Salt stress (0.3 M NaCl) and drought stress (0.6 M mannitol) induced in the knock-out mutant transient stromal [Ca^{2+}] increases characterized by similar kinetics, with Δ[Ca^{2+}] peaks of 2.41 ±0.25 μM, n=5 (Fig. 2a) and 2.67 ± 0.24 μM, n=5 (Fig. 2b), respectively. These values were found to be significantly different (p<0.05) from the Δ[Ca^{2+}] recorded in the wild-type (Supplementary Fig. S1), which were characterized by comparatively lower amplitutes of the Ca^{2+} peaks (1.46 ± 0.22 μM, n=6 for salt stress and 1.48 ± 0.21 μM, n=7 for drought stress). A statistically significant difference was also observed in the magnitude of the Ca^{2+} change triggered in response to cold shock (3 volumes of ice-cold H_2O) with average Δ[Ca^{2+}] peaks of 3.28 ± 0.76 μM, n=5, in the knock-out mutant and 1.11 ± 0.14 μM, n=7 in the wild-type (Fig. 2c and Supplementary Fig. S1).

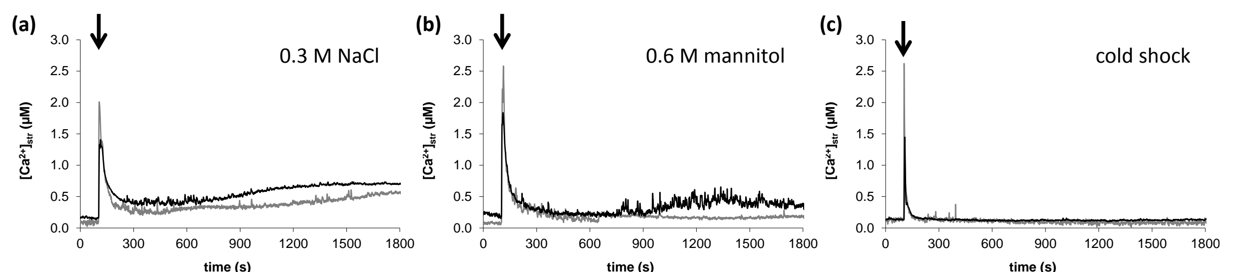
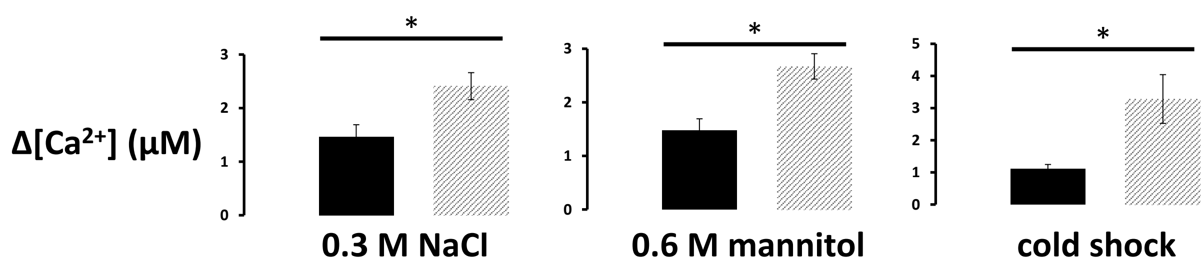


Fig. 2 Comparison between stromal Ca^{2+} dynamics in *Arabidopsis* wild-type (black trace) and plastidial MCU knock-out (grey trace) plant lines, both expressing aequorin in the plastid stroma. Seedlings were challenged (after 100 s, arrow) with different abiotic stimuli, as specified in each panel. These and the following Ca^{2+} traces are representative of at least 5 independent experiments, that give similar results.



Supplementary Fig. 1 Statistical analysis of Δ[Ca^{2+}] recorded in *Arabidopsis* wild-type (black bar) and MCU knock-out (gray bar) lines, stably expressing aequorin in the chloroplast stroma in response to various abiotic stimuli. Asterisks indicate statistically significant differences (p<0.05).

In response to oxidative stress (10 mM H_2O_2) even more remarkable differences were observed between the stromal Ca^{2+} trace recorded in the MCU knock-out line and wild-type, respectively. Indeed, the stromal Ca^{2+} response in the mutant was found to be characterized by a steady-state level of much higher amplitude (0.74 ± 0.13 µM after 700 s, n= 5) than that recorded in the wild-type (0.29 ± 0.03 µM after 700 s, n=5) (Fig. 3a). The major participation of the plastidial MCU isoform in oxidative stress-activated Ca^{2+} fluxes was confirmed by a pharmacological approach, using the MCU specific inhibitor NecroX-5 (Thu et al., 2012). The stromal Ca^{2+} change recorded in the wild-type after 30 min pre-treatment with 50 µM Necrox-5 followed by challenge with 10 mM H_2O_2 displayed a dynamic (Fig. 3b) that closely resembled that one recorded in the MCU knock-out line in response to the same stimulus, in the absence of the Ca^{2+} channel blocker (Fig. 3a).

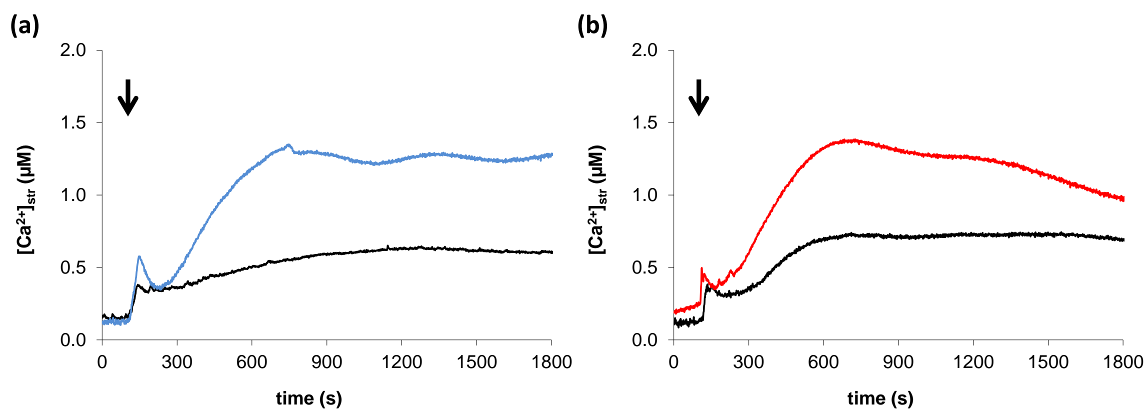


Fig. 3 Monitoring of stromal $[Ca^{2+}]$ in *Arabidopsis* transgenic MCU knockout and wild-type lines in response to oxidative stress, and effect of the MCU inhibitor NecroX-5 on the Ca^{2+} response. (a) Wild-type (black trace) and MCU knock-out (blue trace) seedlings were challenged with 10 mM H_2O_2. (b) Wild-type seedlings were pre-treated (red line) or not (black line) with 50 µM Necrox-5 and subsequently challenged with 10 mM H_2O_2. Arrows indicate stimulus injection. Traces are representatives of at least 5 independent experiments.

These experiments were carried out by using both *Arabidopsis* MCU knock-out sub-lines expressing stromal aequorin (#6 and #7), with very similar results. These unexpected results, demonstrating a significantly higher overall stromal Ca^{2+} response in the knock-out line than in the wild-type, open up a new scenario, where the plastidial MCU homologue may have an intra-chloroplast localization at the thylakoid membrane, rather than at the plastid envelope. Further investigations are needed to ascertain the intracellular localization of the plastidial MCU homologue (see Discussion Section).

In order to carry out Ca^{2+} measurement assays not only in the stroma but also in the thylakoid lumen of the plastidial MCU knock-out mutant, *Arabidopsis* plants of the MCU knock-out line were transformed with the construct encoding YFP-aequorin targeted to the thylakoid lumen (see Chapter 5). After screening of the T1 generation on 50 µg/ml

kanamycin, the expression and correct localization of the aequorin chimera in the T1 transformants was verified (Fig. 4). Experiments are currently under way in our laboratory to analyze differential Ca^{2+} signatures between wild-type and knock-out lines expressing aequorin in the thylakoid lumen.

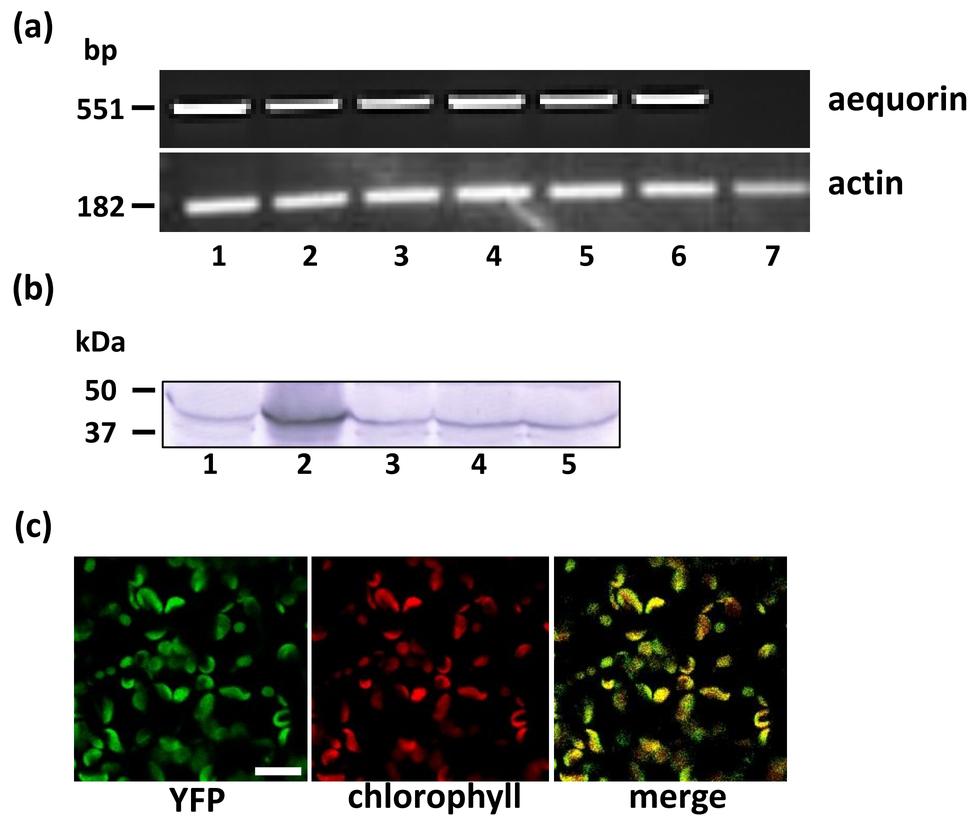


Fig. 4 Aequorin expression and intracellular localization in *Arabidopsis* MCU knock-out lines stably transformed with the construct encoding a YFP-aequorin chimera targeted to the thylakoid lumen. Analyses were carried out by RT-PCR (a), immunoblot (b) and confocal microscopy (c). Examples of some positive transformants of the T1 generations (lanes 2-6) are shown in (a). A wild-type line (lane 7) and the line expressing aequorin in the chloroplast stroma (lane 1) were used as negative and positive control, respectively. Bar, 10 µm.

Discussion

The preliminary results presented in this Chapter provide evidence for the participation of an *Arabidopsis* MCU homologue localized in plastidial membranes in organellar Ca^{2+} dynamics in response to environmental stimuli. Its role in mediating Ca^{2+} fluxes in the chloroplast was confirmed by both a genetic approach, by using a knock-out mutant line lacking the putative Ca^{2+}-permeable transporter, as well as a pharmacological approach using Necrox-5, a specific inhibitor of the mammalian MCU (Thu et al., 2012). Two independent MCU knock-out sub-lines stably expressing stroma-targeted aequorin were isolated, and appropriate analyses to confirm the expression and correct localization

of the recombinant Ca^{2+} reporter were carried out. Ca^{2+} measurement assays demonstrated the occurrence of stromal [Ca^{2+}] increases characterized by higher amplitudes in the MCU ko plant line, in comparison with the wild-type, in response to different stimuli (Fig. 2-3). These unexpected results suggest that the plastidial MCU homologue may be involved in the dissipation of stromal Ca^{2+} signals - *via* mediation of Ca^{2+} influx into the thylakoid lumen - rather than in their generation. Pharmacological analyses carried out by pre-treating wild-type seedlings with 50 μM Necrox-5, a specific inhibitor of MCU, and subsequently challenging the plants with oxidative stress, confirmed the results obtained with the genetic approach (Fig. 3b).

Based on these data, the intra-chloroplast localization of this Ca^{2+} transporter may be at the thylakoid membrane, instead of the plastid envelope, as it could have been envisaged by similarity to the mammalian MCU, which is located at the inner membrane of mitochondria (Murgia & Rizzuto, 2015). In fact, a vesicle transport system from the inner envelope membrane has been proposed to be involved in thylakoid biogenesis (Vothknecht & Soll, 2005). Moreover, the shift from a non-thylakoid to a thylakoid location seems to have repeatedly occurred for different transport proteins during evolution (Pfeil et al., 2014).

Current research activities, carried out in collaboration with Szabò's team, are being focused on the elucidation of the sub-chloroplast localization of the plastidial MCU homologue. Two approaches are being pursued, *i.e*: i) engineering a DsRed-tagged version of the plastidial MCU to be used in confocal microscopy analyses; ii) the production of a specific anti-plastidial MCU antibody, to be used in membrane fraction studies as well as immunocytochemical studies. Moreover, Ca^{2+} measurement assays with the MCU knock-out line transformed with the construct encoding aequorin targeted to the thylakoid lumen, currently ongoing in our laboratory, should help to elucidate whether the plastidial MCU homologue is actually involved in Ca^{2+} fluxes between the stroma and the thylakoid lumen, rather than in in/out fluxes from/to the cytosol.

This work, which at the time of writing this PhD thesis is not yet finished, should shed light on novel aspects regarding the Ca^{2+} transport mechanisms that allow Ca^{2+} to flow through sub-chloroplast membranes. Unveiling the chloroplast Ca^{2+} homeostatic apparatus is of high relevance to understand the role of Ca^{2+} in regulating fundamental processes taking place in this organelle, primarily related to photosynthesis (Hochmal et al., 2015).

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CHAPTER 7.

Ca^{2+}-mediated perception of environmental stimuli in the freshwater unicellular alga *Chlamydomonas reinhardtii*

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Abstract

Plants use calcium (Ca^{2+}) as an intracellular messenger to transduce a wide variety of environmental stimuli, both biotic and abiotic. The involvement of variations in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in response to such stresses is well established in land plants, but there is still a lack of knowledge regarding the role of this ion in green microalgae, such as the freshwater alga *Chlamydomonas reinhardtii*. Recent evidence is available concerning the function of Ca^{2+} in different flagellar processes (*i.e.* excision and intraflagellar transport), but there are no clues about $[Ca^{2+}]_{cyt}$ dynamics in response to environmental stimuli in this model organism. No evidence is currently available regarding the conservation of Ca^{2+} signalling in the green lineage in response to external stimuli. In this work we provide novel aspects for the ability of *Chlamydomonas* in generating specific $[Ca^{2+}]_{cyt}$ variations in response to salt and hypoosmotic stresses, but not to hyperosmosis and oxidative conditions. Moreover, $[Ca^{2+}]_{cyt}$ elevations induced by salt stress and hypoosmotic shock exhibited distinct spatial and temporal characteristics, which may contribute to specificity in the respective Ca^{2+} signalling pathways. We conclude hypothesizing that the typical $[Ca^{2+}]$ elevations observed in *Chlamydomonas* may be due to the different Ca^{2+} signalling toolkits found in algae, and physiological differences with higher plants might have taken to the evolution of different mechanisms to cope with environmental stresses.

Introduction

Calcium (Ca^{2+}) is well known to be involved in the intracellular transduction of a plethora of environmental stimuli in a wide variety of organisms, covering all branches of evolution from unicellular prokaryotes to multicellular eukaryotes (Plattner & Verkhratsky, 2015). Compared to other living beings, land plants are sessile organisms that have to adapt to environmental changes without the possibility to move. In plants, Ca^{2+} is one among different intracellular messengers and it is used to transduce a broad variety of abiotic and biotic stresses that have been shown to evoke specific intracellular spatio-temporal Ca^{2+} signals, which are further transduced by Ca^{2+} sensor proteins into transcriptional and metabolic responses (Dodd et al., 2010). The involvement of Ca^{2+} in the perception of stimuli like salt stress and drought stress (Knight et al., 1997), hypoosmotic stress (Takahashi et al., 1997) and oxidative stress (Evans et al., 2005) is well established in higher plants.

In contrast to plants, little evidence is available about the involvement of Ca^{2+} in the perception of the extracellular environment in algae. Ca^{2+} signalling in this group has been examined in diatoms, green algae and brown macroalgae. For example, the marine diatom *Phaeodactylum tricornutum* (a unicellular alga) has shown the ability to respond to hypoosmotic stress, but not to hyperosmotic stress, with a transient elevation in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) (Falciatore et al., 2000). The perception of a hypotonic environment caused a single $[Ca^{2+}]_{cyt}$ increase that peaked after 1 to 2 s and was quickly dissipated in 10 s. The ability of *P. tricornutum* to respond to a second treatment immediately following the initial stimulus was also tested and no desensitization effect was observed. As a matter of fact, no differences in the magnitude of $[Ca^{2+}]_{cyt}$ elevations caused by the first and the second treatment (from 5s to 30min after the first) were detected. Another Stramenopile, the brown macroalga *Fucus serratus*, was found to respond to incrementing degrees of hypoosmosis with increasing variations in $[Ca^{2+}]_{cyt}$ at the stage of embryo (Goddard et al., 2000), and a gradient of $[Ca^{2+}]_{cyt}$ was measured in rhizoids of germinating zygotes of the same organism (Brownlee & Wood, 1986).

Green algae belong, together with higher plants, to the Viridiplantae clade (Leliaert et al., 2012). As these two subgroups of photosynthetic organisms share a common autotrophic ancestor, the study of the involvement of Ca^{2+} in the perception of environmental stimuli in green algae is of great interest from an evolutionary point of view, *i.e.* which responses are specific to organisms adapted to the terrestrial environment. The role of Ca^{2+} in response to external stimuli has been observed in the coccal green

freshwater alga *Eremosphaera viridis* as well, where a "light-off" stimulus induced a single quite long-lasting transient elevation in the $[Ca^{2+}]_{cyt}$ (Bauer et al., 1997). The increase started a few seconds after "light-off" and the dynamic lasted nearly 1 min. Variations in $[Ca^{2+}]$ were also detected during the deflagellation process in the soil and freshwater alga *Chlamydomonas reinhardtii.* Wheeler et al. (2008) demonstrated that a rapid elevation in $[Ca^{2+}]_{cyt}$ was observed together with either acid- or external $[Ca^{2+}]$-induced flagellar excision. As these transient $[Ca^{2+}]_{cyt}$ increases had an approximate duration of some seconds, they were much more rapid if compared to those recorded in *E. viridis* and *P. tricornutum*.

Despite the evidence for the involvement of Ca^{2+}, there is a lack of knowledge about the conservation of Ca^{2+} dynamics in response to environmental stresses between green algae and higher plants. As these two groups of organisms have very different physiological aspects but diverged from the same common ancestor between 500 and 700 mya (Lewis & McCourt, 2004), the mechanisms to adapt and respond to the extracellular environment could be either largely conserved or different systems could have evolved. An interesting point that could be addressed is if signalling processes in plants reflect ancestral adaptations to stresses associated with colonisation of the terrestrial environment or more recent innovations have specifically evolved in the land plant lineage. Moreover, the mechanisms responsible for generating Ca^{2+} elevations in *Chlamydomonas* are quite different to land plants. *Chlamydomonas* has a quite different set of Ca^{2+} channels compared to higher plants, as it presents genes encoding voltage dependent Ca^{2+} channels, a mammalian inositol triphosphate homologue and transient receptor potential channels (Pazour et al., 2005; Merchant et al., 2007). All of these appear to have been lost in land plants. On the other hand, other families of Ca^{2+} channels are conserved between higher plants and the unicellular alga, such as cyclic-nucleotide gated channels (which massively expanded in land plants), glutamate receptors and mechanosensitive-like ion channels (Edel & Kudla, 2015).

To shed some light on the conservation of the ability to use Ca^{2+} as an intracellular messenger in the green lineage, the effect of relevant environmental stresses on the freshwater unicellular alga *C. reinhardtii* was considered. All eukaryotes use Ca^{2+} as an intracellular messenger. So the ability to use it is likely to be conserved, but the way in which it is used may not be - *i.e.* differences between stimuli and dynamics of Ca^{2+} elevations. In this thesis the role of plastids in higher plants in handling Ca^{2+} in response to external stresses has been investigated. As no evidence for the involvement of Ca^{2+} in the

perception of environmental stimuli in *Chlamydomonas* is yet available, attention was focused on understanding cytosolic [Ca^{2+}] dynamics of this organism. With this aim, *C. reinhardtii* cells were biolistically loaded with the Ca^{2+}-responsive fluorescent dye Oregon Green-BAPTA in order to monitor variations in $[Ca^{2+}]_{cyt}$ in response to the perception of salt stress, drought stress, oxidative stress and hypoosmotic stress. Variations in $[Ca^{2+}]_{cyt}$ were monitored and recorded using fluorescence microscopy during the perfusion of different stimuli, *i.e.* 150-400 mM NaCl, 200-400 mM mannitol, 5-20 mM hydrogen peroxide and 50 µM Ca^{2+} distilled water. Moreover, the involvement of external Ca^{2+} in internal Ca^{2+} dynamics and a pharmacological approach were considered with the aim of clarifying some of the mechanisms that could be responsible for the observed intracellular Ca^{2+} dynamics. Data presented in this work show that *C. reinhardtii* is able to respond either to salt stress or hypoosmotic shock with specific $[Ca^{2+}]_{cyt}$ elevations for the two different stimuli, but not to drought and oxidative stress. We focused on the hypoosmotic stress to further investigate the nature of the Ca^{2+} signal due to the robustness of the response. The results obtained give new evidence about the ability of *C. reinhardtii* cells to respond to changes in external osmolarity with $[Ca^{2+}]_{cyt}$ dynamics that are different from those observed in any other uni- or multicellular organism.

Materials and methods

Algal strains and growth conditions

The principal wild type *Chlamydomonas reinhardtii* strain used for the present study was CC1021 mt+ (CC1021), but where indicated other strains were used as well. CC1021 were obtained from Dr Stuart Ruffle (University of Exeter, UK), the wild type 137c (CC125 or CCAP 11/32C) and the cell wall deficient 11/32C *cw15*+ mutant (*cw15*) were obtained from the UK Culture Collection of Algae and Protozoa (CCAP) while the wall deficient strain CC3395 cwd *arg7-8* mt- (CC3395) was purchased from the Chlamydomonas Resource Center (University of Minnesota, St. Paul, MN, USA). Cell cultures were grown in liquid medium containing standard Tris-acetate-phosphate (TAP) medium (Gorman and Levine, 1965) made in MilliQ water (Merck Millipore, Darmstadt, Germany). CC3395 was supplemented with additional arginine. Culture flasks were kept in growing cabinets (Sanyo Versatile Environmental Chamber, Sanyo Co. Ltd., Japan) with a

16h light, 8h dark cycle at light intensity of 105±15μmol m^{-2} s^{-1} and controlled temperature at 23˚C.

Ca^{2+} measurements in response to different environmental stresses

On the day of each experiment cells were loaded with two dyes, the Ca^{2+}-responsive green dye Oregon Green-BAPTA Dextran (OG) (10000 MW) and the Ca^{2+}-unresponsive reference dye Texas Red Dextran (TR) (10000 MW) (Invitrogen Ltd, Paisley, United Kingdom). 40 µg OG and 24 µg TR were previously mixed with 30 mg of 0.6 µm gold microcarriers (Bio-Rad, Hercules, CA, USA). An aliquot of cell culture was centrifuged (400 g, 5 min) and resuspended in the loading buffer (10 mM HEPES pH 7.4, 20 µM K^+ glutamate, 50 mM sorbitol), then cells were spread onto a 0.45 µm nitrocellulose filter (Merck Millipore). Dyes were biolistically loaded using the PDS-1000 delivery system and 1100 psi rupture discs (Bio-Rad) as described by Wheeler et al. (2008). Samples were subsequently resuspended in TAP medium and left for 2h in the growing chamber to recover. As cells tend to lose flagella because of the biolistic loading, after 2h of recovery a mixture of flagellated and deflagellated cells could be found in the suspension. With the aim to increase the variability and to investigate if flagella are involved in the perception of the stress, both cells with and without flagella were used for the experiments.

Prior to imaging, cells were resuspended in the Chlamydomonas Assay Buffer (CAB) containing 5 mM Hepes, 1 mM HCl, 1 mM KCl, 0.2 mM EGTA and 0.5 mM $CaCl_2$ and pH was adjusted to 7.4 using using N-methyl-D-glucamine (NMDG) (approximately 200 µM free Ca^{2+}). To perform Ca^{2+} imaging experiments, cells were stuck on 35 mm glass-bottomed dishes (In Vitro Scientific, Pagoda Tree Court, Sunnyvale, CA, USA) coated with 0.01% Poly-D-lysine (Sigma-Aldrich, St Louis, MO, USA). During Ca^{2+} imaging experiments, *C. reinhardtii* cells were perfused firstly with CAB for 30 s, then with the stimulus (150-300 mM NaCl, 200-400 mM mannitol, 5-20 mM hydrogen peroxide) for 1 min, then CAB was perfused again for 1 min. Hypoosmotic experiments were performed by perfusing with CAB firstly for 30 s and then with distilled water containing 50 µM $CaCl_2$ for 1 min 30 s. From now, "distilled water containing 50 µM $CaCl_2$" will be simply called "distilled water". Experiments to investigate the involvement of Ca^{2+} elevations in contractile vacuoles activity were carried out after the adjustment of cells to 100 mM sucrose CAB for at least 1 h. They were then perfused for 10 s with the same medium and shocked with distilled water for 3 min 50 s. In all the experiments the stimulus reached the cells 2-3 s after the beginning of perfusion.

In order to assess if extracellular Ca^{2+} is involved in $[Ca^{2+}]_{cyt}$ elevations in response to hypoosmotic shock, the initial monitoring of intracellular Ca^{2+} dynamics was performed using Ca^{2+}-free CAB and then cells were shocked using distilled water containing 50 µM EGTA.

The involvement of mechanosensitive ion channels in the generation of $[Ca^{2+}]_{cyt}$ elevations after the perception of the stress was taken in account. *C. reinhardtii* cells were pre-treated for at least 10 min with a final concentration of either 5 µM GsMTx4 (Smartox Biotechnology, Rue de la Chimie, Saint-Martin-d'Hères, France) or 10 µM Ruthenium Red (RuR) (Sigma-Aldrich, St Louis, MO, USA) prior to the perfusion with distilled water or distilled water containing 10 µM RuR respectively.

Perfusion was performed using gravity flow from multiple taps with an approximate flow rate of 3 ml min^{-1} and a suction system removed excess liquid from the 35 mm imaging dish.

Epifluorescence microscopy was performed at room temperature using a Nikon Eclipse Ti with a 100x, 1.49 NA oil immersion objective and an EM-CCD camera (Photometrics Evolve, Tucson, AZ, USA). TR was excited using a 561 nm laser (Coherent, Santa Clara, CA, USA) with an emission of 575-625 nm, while OG was excited using a 488nm laser (Coherent) with an emission of 500-550 nm. Both OG and TR were excited simultaneously and a dual-view beam splitter device was used to detect emission. Images were captured using NIS-Elements v3.1 at either 200 ms for 2 min experiments or at 500 ms for longer experiments. Elevations in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) were identified by calculating the mean pixel intensity of defined regions of interest within *C. reinhardtii* cells for each time point, followed by the generation of a fluorescence ratio between OG and TR (see below). Contractile vacuoles activity was detected in the same way. Ca^{2+} dynamics observed in cells after the perception of salt and hypoosmotic stress were processed using ImageJ. OG and TR values were processed with OriginPro 2015 software (OriginLab Corporation, Northampton, MA, USA) to give OG/TR ratios. Ratio traces were smoothed using a Savitsky-Golay filter and a baseline trace was also created using the same software. Ratio traces were subtracted by their respective baseline traces to give the relative increase in fluorescence. A threshold equivalent to 3% was chosen to discriminate between signal noise and Ca^{2+} elevations. Any increases in OG fluorescence of ≥3% that lasted more than 1 frame (200 ms) were counted as Ca^{2+} elevations.

In vitro calibration of OG fluorescence

An *in vivo* calibration was not performed as the Ca^{2+}-ionophores do not work particularly well in *Chlamydomonas* (Braun & Hegemann 1999). An *in vitro* calibration was therefore performed to assess how changes in dye fluorescence relate to changes in $[Ca^{2+}]$. Oregon Green-BAPTA Dextran fluorescence was calibrated using the Calcium Calibration Buffer Kit #1 (Life Technologies Ltd, Paisley, UK), using 0 and 10 mM Ca^{2+} EGTA buffers. Dye fluorescence was measured by epifluorescent microscopy whilst increasing free $[Ca^{2+}]$ from 0 to 39 µM. The OG calibration worked well, and produced the expected linear response on a log/log graph. This result (main graph, Fig. 1) is displayed as fluorescence on $[Ca^{2+}]$ to demonstrate that this relationship is not linear. If a resting $[Ca^{2+}]_{cyt}$ of around 100 µM is assumed, a 40% increase in dye fluorescence equates to a $[Ca^{2+}]_{cyt}$ of 600 µM. However, as we were unable to accurately determine the resting $[Ca^{2+}]_{cyt}$, the results have been presented as changes in dye fluorescence rather than changes in $[Ca^{2+}]_{cyt}$.

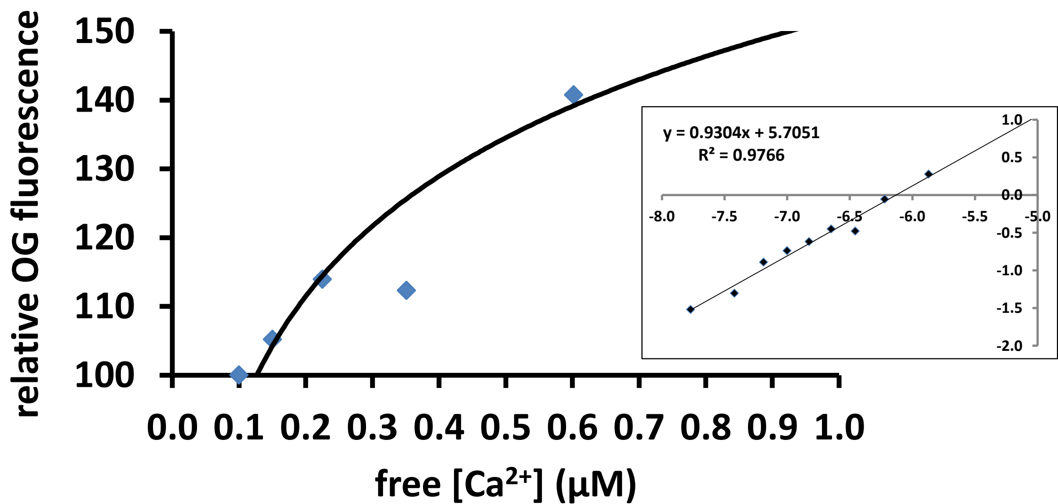

Fig. 1 Non-linear relation between OG fluorescence and free $[Ca^{2+}]$. OG calibration curve has shown how the fluorescence of the Ca^{2+} responsive dye is not directly proportional with the free Ca^{2+} concentration, but increases less at low free $[Ca^{2+}]$. The performance of Oregon Green BAPTA is shown in the log plot to demonstrate that the dye behaves as expected. X and y axis are $\log_{10}$ free $[Ca^{2+}]$ and $\log^{10}$ $\{(F - F_{min})/(F_{max} - F)\}$ (F: fluorescence recorded at the different free $[Ca^{2+}]$; F_{min}: fluorescence recorded in absence of free Ca^{2+}; F_{max}: fluorescence recorded in saturating Ca^{2+} conditions).

Statistical analysis

Data obtained from all treatments are shown as average ± standard error. Statistical significance ($P < 0.05$) between averages was calculated using the Student's *t*-test.

Results

Chlamydomonas reinhardtii strain CC1021 was used in the present study to test whether the perception of relevant environmental stresses led to elevations in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$). With this purpose, cells loaded with the Ca^{2+}-responsive green dye Oregon Green-BAPTA Dextran (OG) and the Ca^{2+}-unresponsive reference dye Texas Red Dextran (TR) were resuspended in the Chlamydomonas Assay Buffer (CAB) and then shocked with NaCl, mannitol, H_2O_2 and distilled water, and $[Ca^{2+}]_{cyt}$ dynamics were monitored for all the duration of each experiment using epifluorescence microscopy.

Drought and oxidative stress do not induce any $[Ca^{2+}]_{cyt}$ elevation in the freshwater alga *C. reinhardtii*

The treatment of *Chlamydomonas* cells with different concentrations of mannitol (200, 300 and 400 mM), to mimic drought stress, and H_2O_2 (5, 10 and 2 0mM), to induce oxidative stress, did not induce any significant $[Ca^{2+}]_{cyt}$ elevation within the time period of observation (2 min) (Fig. 2A-B). A higher concentration of mannitol, that is 600 mM, is usually used to monitor its effects on intracellular Ca^{2+} dynamics in higher plants, but *Chlamydomonas* has a less rigid cell wall than land plants. We noticed that in 200 mM mannitol *Chlamydomonas* cells shrunk in volume and this effect increased with an increasing concentration of the stimulus, preventing the use of higher concentrated solutions. None of the cells examined (n=18) showed any increase in $[Ca^{2+}]_{cyt}$ that could be related with the perception of the hyperosmotic stress, as no changes in $[Ca^{2+}]$ after the addition of the stimulus were recorded (Fig. 2A).

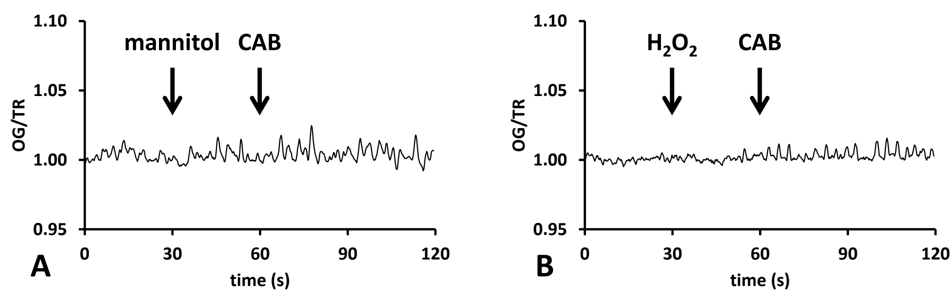

Fig. 2 $[Ca^{2+}]_{cyt}$ measurements in *C. reinhardtii* cells in response to hyperosmotic and oxidative stress. (A) Mannitol, that mimics drought stress, did not induce any $[Ca^{2+}]_{cyt}$ elevation in *Chlamydomonas* during the 30s-perfusion. The trace showed was recorded after the perfusion of 300 mM mannitol and it is a representative for 18 independent experiments. (B) Oxidative stress induced by using three different concentrations of hydrogen peroxide (5, 10, 20 mM) did not trigger any $[Ca^{2+}]_{cyt}$ dynamic in the considered time. A longer exposure (5 min) of cells to the stress at the highest concentration was also tested, but no differences were recorded. The trace showed in fig. B was recorded after the perfusion with 20 mM H_2O_2 and it is a representative for 18 independent experiments. The arrow on the left in each figure indicates the timing of perfusion of mannitol and hydrogen peroxide in A and B respectively, while the one on the right pinpoints the return to perfusion of CAB (assay buffer).

Similar results were found after the perfusion of cells with three different concentrations of hydrogen peroxide. Higher plants respond to reactive oxygen species with low but long lasting Ca^{2+} elevations that can last more than 3000 s (Evans et al., 2005), but in *Chlamydomonas* no Ca^{2+} dynamics were detected in any of the 18 tested cells with the three H_2O_2 considered concentrations (Fig. 2B). These results show that, within the tested conditions, the perception of both drought and oxidative stress do not seem to be conserved between the unicellular freshwater alga *Chlamydomonas* and higher plants.

Salt stress induces single $[Ca^{2+}]_{cyt}$ elevation that spreads in a wave-shape through the cytosol

In intact whole *Arabidopsis* seedlings, salt stress (333 mM NaCl) induces a single rapid $[Ca^{2+}]_{cyt}$ elevation that reaches the maximum in about 5 s and returns to resting levels after about 20 s (Knight et al., 1997). This increase was partially inhibited by either lanthanum chloride or EGTA, indicating the involvement of extracellular Ca^{2+} in this response. However, the response was not totally abolished by either of these inhibitors, suggesting that other intracellular Ca^{2+} stores could be involved in the salt-induced $[Ca^{2+}]_{cyt}$ responses in *Arabidopsis*. With the aim to compare the conservation of Ca^{2+} signalling in response to salt stress between different photosynthetic organisms, we monitored $[Ca^{2+}]_{cyt}$ dynamics in response to a range of NaCl concentrations (150, 200, 300 and 400 mM) in *C. reinhardtii*. Our results show that 150 and 200 mM NaCl did not induce a consistent Ca^{2+} signalling response, with only one cell exhibited a single Ca^{2+} elevation with the higher concentration (n=11 and 12 respectively). More consistent results were obtained with the perfusion of 300 mM NaCl (Fig. 3A). 9 cells out of 61 showed a single rapid $[Ca^{2+}]_{cyt}$ spike that peaked at an average of 7.04±0.73 s after the beginning of the perfusion of the stimulus and reached a magnitude of 8.93±0.97% over basal level. The elevation lasted for 2.04±0.33 s (over the 3% detection threshold), and returned to the basal level within 5 s. In order to examine whether the Ca^{2+} increase was dose-dependent, cells were treated with 400 mM NaCl. Unfortunately, at this concentration cells shrunk immediately after the stimulus reached them, making the detection of Ca^{2+} dynamics impossible. It is not clear why only a small proportion of the cells (c. 15%) exhibit a $[Ca^{2+}]_{cyt}$ elevation in response to 300 mM NaCl. The possibility that different cells do not respond in the same way to changes in external ionic concentration has to be taken in account as this eventuality has already been observed (Allen et al., 2001). Spontaneous

[Ca^{2+}] oscillations were observed in *Arabidopsis* guard cells when the external osmolarity was dropped, but [Ca^{2+}] elevations were observed only in 32% of the cells.

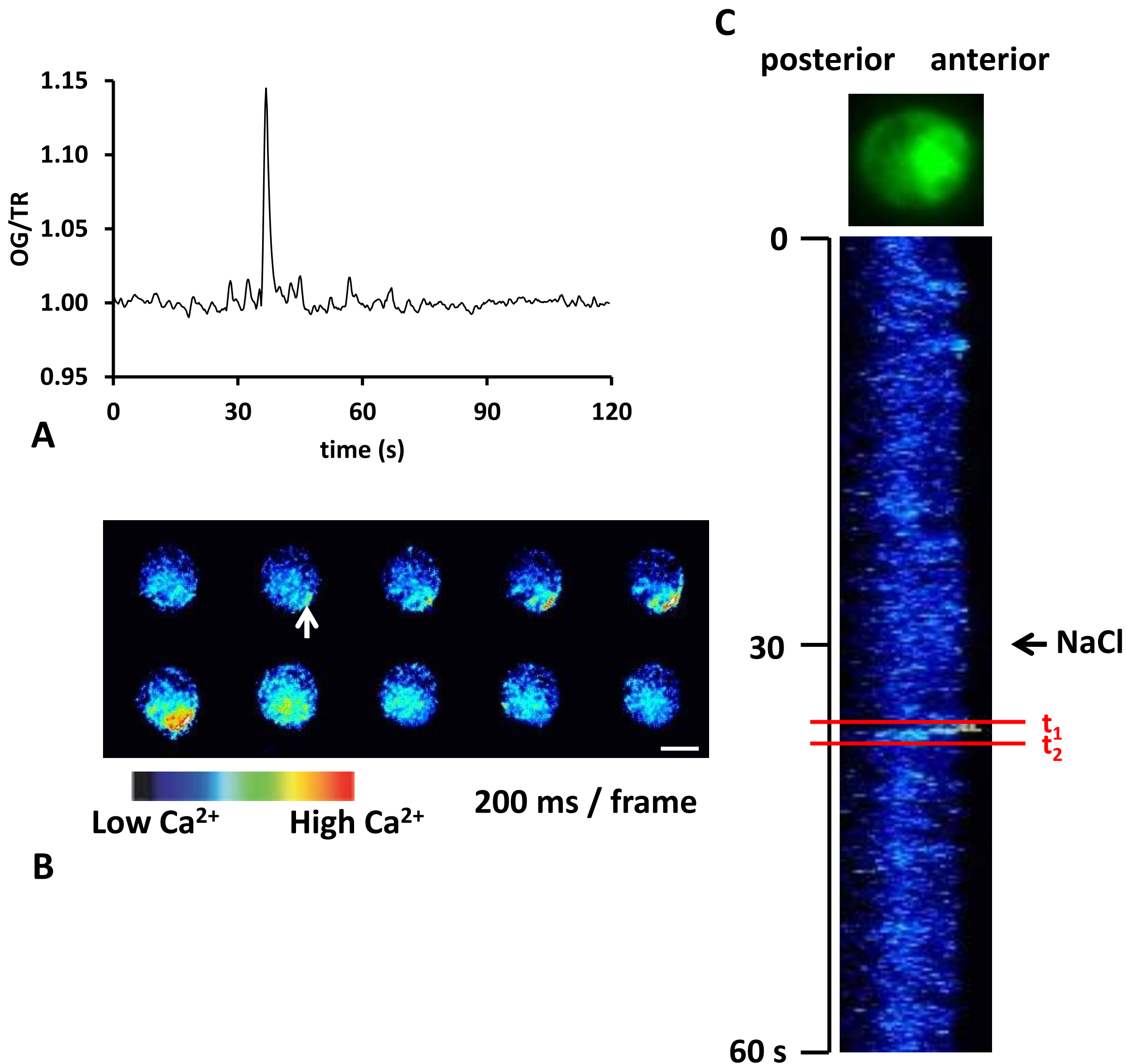

Fig. 3 Detection of a single [Ca^{2+}]$_{cyt}$ elevation in salt-stressed *Chlamydomonas* cells. (A) NaCl induced a single fast [Ca^{2+}]$_{cyt}$ spike that peaked about 7s after the perception of the stimulus and returned to the basal level in less than 5s. One trace is shown as an example representing 9 independent experiments. (B) This sequence of frames taken every 200ms shows that the initial Ca^{2+} elevation at the apex of the cell (white arrow) spreads through the cytosol in about 2s. White bar = 8 µm. (C) The first apex-localized Ca^{2+} elevation occurs at the anterior side of the cell just few seconds after the perfusion of NaCl (black arrow) and it is then spread towards the posterior part. The variation in [Ca^{2+}] in the space during the considered time lapse is pointed out by red lines t_1 and t_2, where t_2=t_1+1400 ms.

Spatial analysis of cells exhibiting Ca^{2+} elevations demonstrated that an early apex-localized increase in OG fluorescence was observed in 5 of them, that than typically spread through the cytosol towards the posterior side of the cell in a wave-shape within about 2 s (Fig. 3B-C). In animal cells, the role of inositol trisphosphate receptor (IP_3R) in producing fast Ca^{2+} waves through the cell via Ca^{2+}-induced Ca^{2+} release (CICR) from the

endoplasmic reticulum is well established (Jaffe, 2010), and evidence of its role in generating Ca^{2+} waves in the marine alga *Fucus serratus* was also found (Goddard et al., 2000). As the presence of an IP_3R homologue has been found in *Chlamydomonas* (Merchant et al., 2007), it is possible that the Ca^{2+} wave that was observed in *C. reinhardtii* in response to salt stress is caused by an initial localized Ca^{2+} increase that induces a CICR from internal compartments, such as the endoplasmic reticulum.

Hypoosmotic stress induces multiple Ca^{2+} elevations in *C. reinhardtii* cells

Many studies on different uni- and multicellular organisms, such as animal epithelial cells, tobacco, the marine diatom *Phaeodactylum tricornutum*, the protozoan *Trypanosoma cruzi* and the yeast *Schizosaccharomyces pombe*, have identified that Ca^{2+} is involved in the perception of extracellular hypotonic conditions (Christensen, 1987; Takahashi et al., 1997; Falciatore et al., 2000; Rohloff et al., 2003; Nakayama et al., 2012). As a matter of fact, a single or long lasting intracellular Ca^{2+} elevation was detected in all the considered organisms using different Ca^{2+} probes (*i.e.* aequorin, fura-2 and Cameleon-nano15) in response to the sensing of a low-solute environment.

Our initial results showed that *C. reinhardtii* is able to evoke repetitive $[Ca^{2+}]_{cyt}$ elevations in response to hypoosmotic stress (Fig. 4). The shock consisted in changing environmental conditions moving from the CAB buffer used for Ca^{2+} measurements assays to distilled water. 70.97% of the cells (total number=31) responded with at least one $[Ca^{2+}]_{cyt}$ elevation. The average number of peaks per cell in the 60 s period following addition of the stimulus was 3.93±0.47 and the average duration of the Ca^{2+} elevation (above the threshold value 3%) was 1.91±0.50s (n=14). The amplitude of the peaks is variable, with higher increases at the beginning of the response and lower spikes later on, with a mean amplitude of 6.08±0.50% (n=14). The number of cells used for these statistics is lower than the total amount in which a Ca^{2+} increase was recorded because not all of them were suitable for detailed analysis.

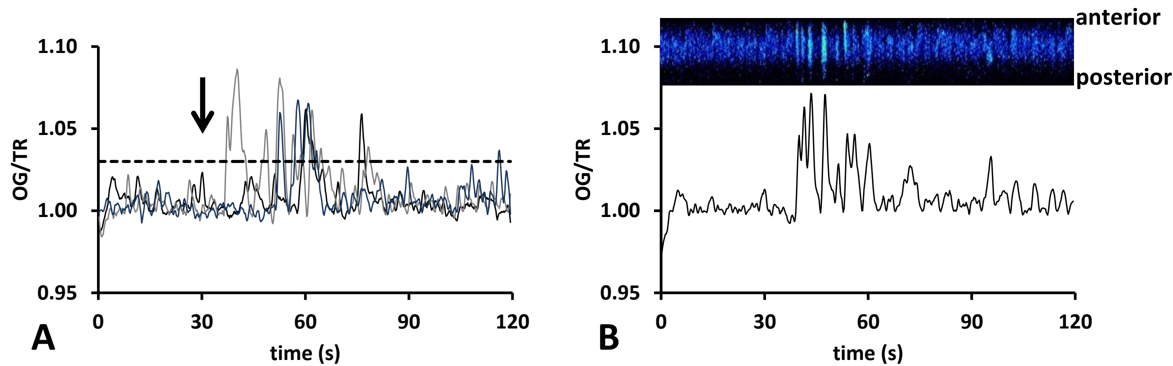

Fig. 4 Multiple cytosolic Ca^{2+} elevations in the freshwater alga *C. reinhardtii* in response to the hypoosmotic stress. (A) Three OG/TR ratio traces (overlaid) obtained from independent experiments are shown (representative of 22 cells out of 31 that exhibited Ca^{2+} elevations). Multiple $[Ca^{2+}]_{cyt}$ elevations were detected in the majority of the cells (nearly 80%) after the perception of the hypoosmotic stimulus with a typical pattern for every cell. The dashed line delimits the 3% value chosen as the threshold to discriminate between signal noise and Ca^{2+} elevations. The black arrow indicates the beginning of the stimulus perfusion. (B) Epifluorescent microscopy of cytosolic Ca^{2+} elevation at high temporal resolution (200ms per frame). The profile in the black box shows the OG fluorescence across a section of the cell projected over time. Repetitive Ca^{2+} elevations can be observed following hypoosmotic shock. The graph shows a representative Ca^{2+} dynamic trace obtained from the mean fluorescence intensity of the full cell.

As can be seen from fig. 4A, the first Ca^{2+} elevation does not occur instantly after the stress had reached the cells, but there was a delayed response as the first peak could be detected only 15.93±1.53s after the beginning of the perfusion of the stimulus. These results demonstrate that multiple $[Ca^{2+}]_{cyt}$ elevation are evoked in *C. reinhardtii* cells in response to the perception of a diluted extracellular environment. Moreover, to further prove that *Chlamydomonas* cells are able to respond more than once to the same stimulus and to demonstrate that is the perception of the water itself that causes $[Ca^{2+}]_{cyt}$ increases, we carried out a series of experiments in which cells were exposed a second time to water. To do this, cells that responded the first time to the hypoosmotic shock were perfused with CAB for 10min to take them back to the original osmotic condition and then hypoosmotic stress was applied again for 90s. Out of 12 tested cells, 9 responded with multiple $[Ca^{2+}]_{cyt}$ elevations and 5 among them responded again to the same stimulus with at least one $[Ca^{2+}]_{cyt}$ elevation. These results confirm the ability of *C. reinhardtii* to repeatedly perceive a diluted extracellular environment.

Wild type cell-walled *C. reinhardtii* strain CC1021 showed repetitive $[Ca^{2+}]_{cyt}$ elevations in response to the hypoosmotic stress. We decided to further investigate this result, examining if the cell wall could have a role in the perception of the stimulus. Strains CC125 (wild type), CW15 and CC3395 (wall deficient) were loaded with the fluorescent dyes, exposed to the shock from CAB to distilled water, and $[Ca^{2+}]_{cyt}$ dynamics were

monitored and recorded. The evidence obtained with CC1021 was confirmed by CC125 as this strain responded to the water shock with multiple $[Ca^{2+}]_{cyt}$ increases in 53.85% of the cases (n=13), with a very similar pattern when compared to the other wild type. Surprisingly, using the cell wall deficient strains results were completely different as no significant $[Ca^{2+}]_{cyt}$ elevations could be observed in both circumstances (n=11 for CW15 and CC3395).

The absence of extracellular free Ca^{2+} prevents intracellular Ca^{2+} elevations in response to hypoosmotic shock

Several studies on different organisms have indicated that free Ca^{2+} in the extracellular medium is essential for $[Ca^{2+}]_{cyt}$ elevations in response to various environmental stimuli, included the hypoosmotic shock (Takahashi et al., 1997; Rohloff et al., 2003; Nakayama et al., 2012). In order to determine if an influx of Ca^{2+} from the extracellular environment is responsible for intracellular $[Ca^{2+}]$ elevations observed in *C. reinhardtii* in response to the hypoosmotic shock, cells were briefly acclimatized in Ca^{2+}-free CAB prior to experiments. Subsequently, a hypoosmotic shock in absence of extracellular free Ca^{2+} was applied using distilled water containing 50 μM EGTA. As expected in the case that external Ca^{2+} was needed for $[Ca^{2+}]_{cyt}$ elevations after the perception of the abiotic stress, very few $[Ca^{2+}]_{cyt}$ elevations were recorded. A single late $[Ca^{2+}]_{cyt}$ elevation was observed in two cells (out of 21), but the timing of these events is consistent with spontaneous $[Ca^{2+}]_{cyt}$ elevation elevations. In fact, no cells showed multiple Ca^{2+} elevations and no one exhibited Ca^{2+} elevations within 60s of the stimulus. The proportion of cells that responded did not reach 10% of the total (2 out of 21) (Fig. 5A). This value, compared with the percentage of cells that showed Ca^{2+} peaks in normal conditions (71%) underlines an approximate reduction of 60%. Because of this low number of responding cells, it was impossible to obtain significant results from the statistical analysis. Anyway, the average number of peaks in the cells that responded (1.50±0.50), the amplitude of the first peak (4.26±0.84%) and its duration over the threshold (1.50±0.70s) were reduced in comparison with normal Ca^{2+} conditions. An example of the observed situation is reported in fig. 5B, where the representative traces for the Ca^{2+}-free condition show no significant Ca^{2+} increases, whereas multiple elevations were detected in presence of extracellular Ca^{2+}.

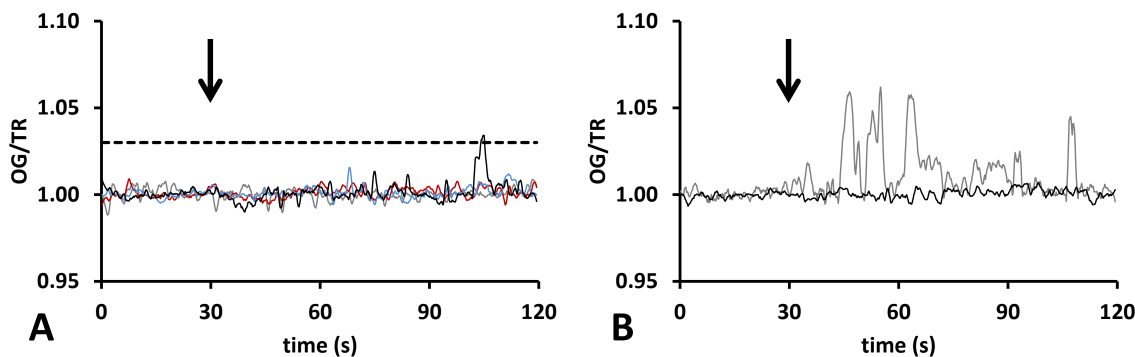

Fig. 5 Effect of extracellular free Ca^{2+} availability on $[Ca^{2+}]_{cyt}$ elevations. (A) Cytosolic Ca^{2+} elevations in response to the hypoosmotic stress (black arrow) are absent or dramatically reduced in the absence of external free Ca^{2+}. The reduction can be observed both in the number of the elevations and their amplitude. The late timing of the only Ca^{2+} elevation (black trace), suggests that it may be a spontaneous Ca^{2+} increase. Ca^{2+} dynamics obtained from four independent experiments are shown as representatives of 21 cells. The dashed line delimits the 3% value chosen as the threshold to discriminate between signal noise and Ca^{2+} elevations. (B) Comparison with the result obtained in normal Ca^{2+} conditions. *C. reinhardtii* cells adapted to Ca^{2+}-free CAB and shocked with distilled water + 50 µM EGTA do not display cytosolic Ca^{2+} elevations in response to the stress. One trace for each condition (black: 0 µM extracellular Ca^{2+}; grey: 50 µM Ca^{2+}) is shown as representative for the two sets of cells.

These results show that extracellular free Ca^{2+} is required to trigger intracellular Ca^{2+} elevations in *C. reinhardtii* in response to a hypotonic environment.

The use of mechanosensitive ion channel blockers reduces the average number of $[Ca^{2+}]_{cyt}$ elevations and their amplitude

When the hypoosmotic shock was applied to *Chlamydomonas* cells, we noticed that in most of the cases they underwent to some event that caused a slight movement of the cell on the dish. This behaviour was observed both in wild type cells with and without flagella and either in presence or absence of Ca^{2+} in the extracellular environment, but not in cell wall deficient strains. As neither flagella nor Ca^{2+} elevations seemed to be responsible for such movement, we hypothesized that the movement was induced by a swelling of the cell caused by the sudden dilution of the extracellular medium. This contingency was tested, and the measurement of swelling is reported in the following section. Hypooosmotic-induced swelling could result in the activation of mechanosensitive ion channels as was previously observed in yeast (Nakayama et al., 2012). The potential role of stretch-activated channels in the initiation of $[Ca^{2+}]_{cyt}$ elevations in response to hypoosmotic shock was tested using inhibitors targeted to this class of channel. To test the potential involvement of mechanosensitive channels in these dynamics, cells were shocked changing the external environment from CAB to water after a 10-minutes pretreatment with either 5 µM GsMTx4 or 10 µM Ruthenium Red (RuR) and the effect of these

chemicals on $[Ca^{2+}]_{cyt}$ increases was monitored. As expected, both inhibitors affected $[Ca^{2+}]_{cyt}$ dynamics but with different levels of inhibition. Compared to control cells, where 70.97% of them responded, GsMTx4 reduced significantly the number of cells that showed a $[Ca^{2+}]$ elevation to 37.5% (total n=16) and lowered the number (Fig. 7A), *i.e.* no multiple Ca^{2+} elevations were observed, and the amplitude (Fig. 7B) of the first Ca^{2+} elevation (6.95±0.64% vs 4.41±0.41%) in cells that responded, as well. GsMTx4 did not have any effect on the timing (Fig. 7C) and the duration (Fig. 7D) of the initial Ca^{2+} elevation. Different results were obtained with 10 µM RuR, which completely abolished all intracellular Ca^{2+} increases (tot n=11) (Fig. 7E).

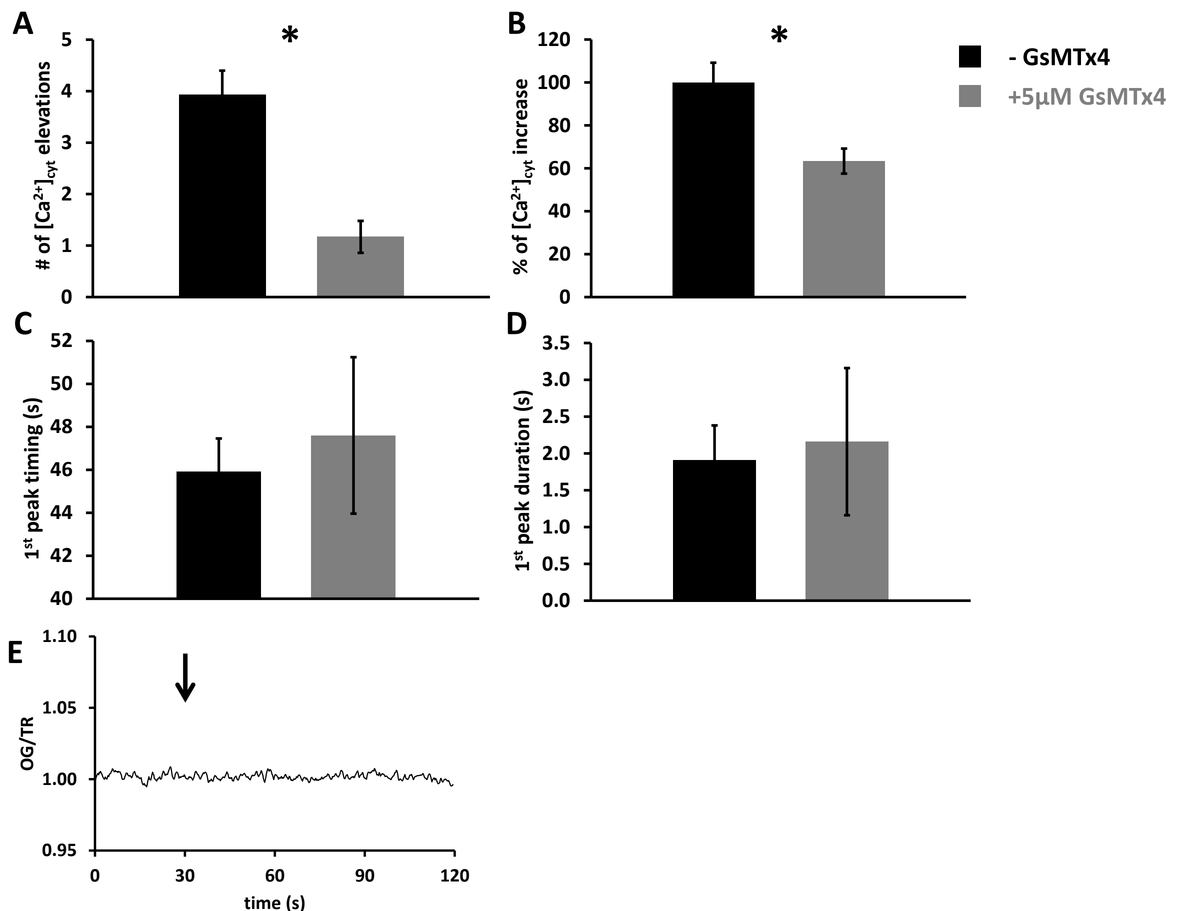

Fig. 7 Mechanosenstive ion channel blockers affect cytosolic Ca^{2+} elevations in response to the hypoosmotic stress. 5 µM GsMTx4 and 10 µM RuR have different effects on $[Ca^{2+}]_{cyt}$ dynamics recorded. Values in **bold** are referred to those that were obtained after the treatment with GsMTx4. Only data obtained from cells that exhibited Ca^{2+} elevations after the treatment with the inhibitor are considered. The number of cells that showed a Ca^{2+} elevation is much lower after GsMTx4 (see the text for percentages). Incubation of *C. reinhardtii* with GsMTx4 caused (A) a significant reduction in the total number of Ca^{2+} elevations and (B) in the amplitude of the first peak in the cells that responded (n=6 out of 16). (C) The timing and (D) the duration over 3% of the 1st peak were not significantly affected by the inhibitor (stimulus added at 30s). Asterisks indicate significant statistical difference (p<0.05) (E) RuR treatment fully inhibited all intracellular Ca^{2+} elevations in response to hypoosmotic shock (black arrow). One trace is shown as representative for 11 independent experiments.

Contractile vacuoles activity is not affected by $[Ca^{2+}]_{cyt}$ elevations

Contractile vacuoles (CVs) are sub-cellular structures that are involved in osmoregulation in many microorganisms, such as *Paramecium*, *Amoeba*, *Dictyostelium*, *Trypanosoma* and *Chlamydomonas*, which are subjected to hypotonic environments (Allen & Naitoh, 2002). CVs collect and expel the excess of water undergoing cycles of diastoles (expansion), in which numerous small vesicles fuse to form the mature contractile vacuole, and systoles (contraction), where the vacuole contacts the plasma membrane, fuses with it and expels its content. So, this process that involves membrane functions – membrane transport, biogenesis, fusion, fission and recycling - has to be in some way finely regulated. A role for Ca^{2+} in biological membrane fusion processes has been established (Burgoyne & Clague, 2003; Starai et al., 2005), but some evidence indicates that several intracellular fusion events do not require calcium (Hay, 2007).

As CVs are therefore important in the response to the hypoosmotic shock, it would be important to investigate *Chlamydomonas* response to that kind of shock in absence of CVs activity. With the aim to investigate if elevations in $[Ca^{2+}]_{cyt}$ are required for the initiation of CVs activity in *Chlamydomonas*, $[Ca^{2+}]_{cyt}$ was monitored in cells that were previously adapted to a slight hypertonic environment to avoid CVs activity. To do so, cells loaded with OG and TR were acclimated to higher osmotic conditions (CAB + 100 mM sucrose) for at least 1h. In these conditions, CV activity was greatly reduced (activity was only detected in 4 cells out of 32). Individual cells responded differently to the hypoosmotic shock (perfusion with 50 μM $CaCl_2$ distilled water), the results are summarised in Table 1. Single or multiple $[Ca^{2+}]_{cyt}$ elevations were recorded in 9 cells out of 32 after the perception of the stress. Interestingly, Ca^{2+} increase events coincided with a clear swelling of cells (fig. 6A, upper and lower panel, respectively) that was observed in all of them except in one. The average volume increase in cells that showed $[Ca^{2+}]$ elevations was 6.47±1.49% (n=8), after the perception of hypoosmotic shock. As it could be expected, CVs started to work during the cellular expansion to expel the excess of water, and reached a rhythmic and regular activity before the end of each experiment. Only in one cell CVs could not be detected. However, a separate group of cells (n=9 of 32) showed a swelling and subsequent CVs activity (fig. 6B, lower panel) without any observable $[Ca^{2+}]_{cyt}$ increase (fig. 6B upper panel). 8 cells did not undergo either to any kind of swelling or initiation of CV activity, and in 2 cells a late $[Ca^{2+}]_{cyt}$ spike without any

other event was detected. As CVs started to be active either with or without $[Ca^{2+}]_{cyt}$ elevations, these results suggest that this function may be induced independently from the $[Ca^{2+}]_{cyt}$ elevations. So, we suggest that, as hypoosmotic stress induced swelling in more than half of the cells, but only a proportion exhibited $[Ca^{2+}]$ elevations, a threshold level of stimulus is required in order to trigger Ca^{2+} spikes.

Cell behaviour	Number of cells
$[Ca^{2+}]$ increase+swelling+CVs activity	9
Swelling+CVs activity	9
CVs from the beginning	4
Late $[Ca^{2+}]$ increase	2
Nothing	8

Tab. 1 The response of *Chlamydomonas* cells after the perception of the hypoosmotic stress (from 100mM sucrose CAB to distilled water).

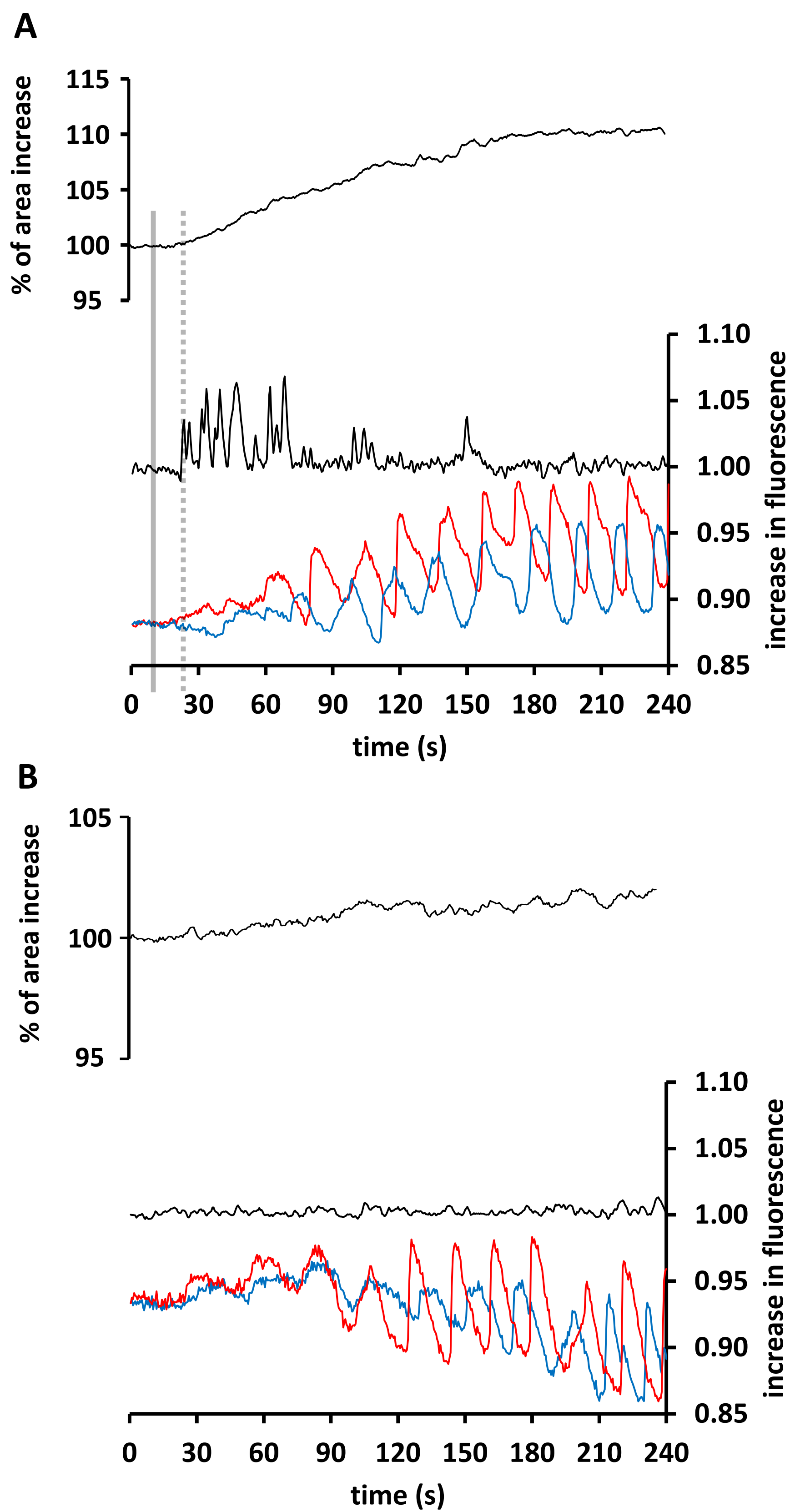

Fig. 6 $[Ca^{2+}]_{cyt}$ elevations in response to hypoosmotic stress are not responsible for initiation of CV activity. (A) A representative cell that exhibited Ca^{2+} elevations, swelling and CVs activity. The increase in cell size due to water influx (upper panel) was observed to overlap with the first Ca^{2+} increase (lower panel, black

trace), pointed out by the vertical dotted line. Area values are used as a measurement of cell size because the software used for the analyses allowed to calculate variations in 2-dimension changes. The onset of stimulus perfusion is showed by the vertical solid line. $[Ca^{2+}]_{cyt}$ dynamics recorded from a swelling cell are shown as OG/TR ratio as representative for 9 independent experiments out of 32. First CVs activities (red and blue lines in the lower panel) were observed later than the beginning of cell swelling, but full, rhythmic and regular activity was recorded when the cell stopped to increase in its volume. (B) A representative cell that did not exhibit any Ca^{2+} elevation, but underwent swelling and showed CVs activity. Cell swelling (black trace in the upper panel) and CVs activity (red and blue traces in the lower panel) were observed in absence of $[Ca^{2+}]_{cyt}$ dynamics as well (black trace, lower panel), showing that Ca^{2+} increases are not needed for CVs activity initiation. These traces are representatives for 9 independent experiments out of 32.

Discussion

Conservation of Ca^{2+} signalling in the green lineage in response to different environmental stimuli

These results show that *C. reinhardtii* strain CC1021 exhibits intracellular $[Ca^{2+}]_{cyt}$ elevations in response to salt stress and hypoosmotic shock, but not to drought and oxidative stress. All these environmental stimuli were tested on *C. reinhardtii* cells to investigate if their Ca^{2+}-dependent signalling mechanism is conserved between the unicellular freshwater green alga and higher plants. Drought stress in the form of 666 mM mannitol has been shown to induce a single $[Ca^{2+}]_{cyt}$ increase in *Arabidopsis* seedlings stably expressing the Ca^{2+} probe aequorin, that returned to basal levels after about 20 min (Knight et al., 1997). Pretreatment with either lanthanum or EGTA, a Ca^{2+} channel blocker and a Ca^{2+} chelator respectively, significantly reduced $[Ca^{2+}]_{cyt}$ elevations, indicating the involvement of extracellular Ca^{2+} in these responses. Our results show that the treatment of *Chlamydomonas* with 300 mM mannitol did not induced any $[Ca^{2+}]_{cyt}$ dynamic in any of the monitored cells, showing that the perception of this stimulus could be different in the alga compared to *Arabidopsis*.

These two organisms have evolved from the same green common ancestor (Edel & Kudla, 2015) and belong to the clade *Viridiplantae*, but they have adapted to live in different environments. These differences led microalgae to evolve distinctive physiological features, such as peculiar cell walls and vacuoles to face changes in external osmotic conditions. The presence of a less rigid cell wall and contractile vacuoles whose activity is adapted following the extracellular environment could have taken *Chlamydomonas* to cope with switches in the surrounding environment in a different way than what arises in higher plants.

Physiological differences could be responsible also for the absence of $[Ca^{2+}]_{cyt}$ dynamics in *Chlamydomonas*, that are not induced after the perception of hydrogen peroxide as an oxidative stress. In higher plants reactive oxygen species (ROS) play an important signalling role in controlling processes such as growth, development, response to biotic and abiotic environmental stimuli, and programmed cell death (Bailey-Serres & Mittler, 2006). For example, ozone fumigation and 20 mM H_2O_2 have shown to induce long lasting $[Ca^{2+}]_{cyt}$ elevations in *Arabidopsis* seedlings, but with typical dynamics for the two stimuli (Evans et al., 2005). As algae have evolved in a different environment, they could have developed quite disparate ways than ROS signalling to face processes like growth and response to some abiotic stimuli. As a matter of fact, treatments up to 5 min with 5, 10 and 20 mM H_2O_2 did not induce any response in terms of changes in $[Ca^{2+}]_{cyt}$ in the freshwater alga *C. reinhardtii*. From these results, it is clear that drought and oxidative stress do not involve any cytosolic Ca^{2+} dynamic in *Chlamydomonas*. This evidence suggests that the two photosynthetic organisms could have evolved different signalling mechanisms, as the typology of stimuli they are exposed to might be peculiar for environment they live in. For example, H_2O_2 is used by higher plants to acclimate to abiotic stresses by cell-to-cell communication as this compound is electrically charged, and can therefore easily travel through biological membranes (Gilroy et al., 2014). On their side, unicellular algae could have evolved different signalling mechanisms to transduce environmental stresses.

Distinct features of *Chlamydomonas* Ca^{2+} signals

Our results show that NaCl and hypoosmotic stress induce different $[Ca^{2+}]_{cyt}$ elevations typical for the two environmental stimuli. The specificity is given by both the number and magnitude of $[Ca^{2+}]_{cyt}$ increases triggered by the shocks. Salt stress induced a single fast Ca^{2+} spike, which was then quickly dissipated. In addition to this, in most of the cells we observed that this Ca^{2+} elevation was characterised by an initial apex-localized Ca^{2+} increase that then spread through the cytoplasm in the form of a Ca^{2+} wave. This event let us to hypothesise that the initial Ca^{2+} spike induced a Ca^{2+}-induced Ca^{2+}-release (CICR) from intracellular Ca^{2+} stores such as the endoplasmic reticulum and could possibly be mediated by inositol trisphospate receptors (IP_3Rs). Regarding the hypoosmotic shock, multiple $[Ca^{2+}]_{cyt}$ elevations of a lower amplitude than the single Ca^{2+} elevation triggered by NaCl were recorded after the perception of an hypotonic environment. These responses were rapid like those induced by NaCl, but repetitive events

were recorded in the majority of the cells. In contrast with sodium-induced $[Ca^{2+}]_{cyt}$ elevations that diffused through the cell, hypoosmotic-induced Ca^{2+} elevations did not show any typical pattern, with the increase occurring simultaneously throughout the cell body. A comparison of these results with the evidence obtained from higher plants shows that the response is conserved between them and *Chlamydomonas*, but not the dynamics. As a matter of fact, $[Ca^{2+}]_{cyt}$ variations in response these two shocks have been well established in land plants, where different Ca^{2+} fluctuations have been recorded. Single long-lasting and dose-dependent $[Ca^{2+}]_{cyt}$ elevations have been attested in response to NaCl in *Arabidopsis* seedlings (Knight et al., 1997). In addition to this, both lanthanum and EGTA reduced the magnitude of Ca^{2+} increases, demonstrating that intracellular Ca^{2+} dynamics were dependent on extracellular Ca^{2+}. A pharmacological approach should be taken in account to prove if external Ca^{2+} is involved in $[Ca^{2+}]_{cyt}$ oscillations in response to NaCl in *Chlamydomonas* as well, and this approach was considered, but due to the infrequent nature of the Ca^{2+} elevations (15%) it might prove difficult to identify the effects of inhibitors. If so, the initial apex-localized $[Ca^{2+}]_{cyt}$ spike should be reduced. Moreover, the involvement of this first increase in inducing CICR from intracellular Ca^{2+} stores could be proved. An additional evidence of the implication of CICR in triggering the observed propagation of Ca^{2+} increase through the cell body could be given by the use of *Chlamydomonas* mutants lacking the IP_3R, as it is well known that IP_3 is involved in the amplification of cytosolic Ca^{2+} signals (Bootman et al., 2002).

The overall response to hypoosmotic shock seems to include a relationship among swelling, mechanosensitive ion channels, Ca^{2+} fluxes and contractile vacuoles

The perception of hypoosmotic stress in higher plants has shown to induce a dramatic increase $[Ca^{2+}]_{cyt}$-dependent luminescence in aequorin-expressing tobacco cell suspension cultures (Takahashi et al., 1997). An immediate, small increase in $[Ca^{2+}]_{cyt}$ was followed by a rapid, large increase starting 35 s after dilution. Luminescence intensity peaked at 70s, rapidly decreased for the next 30 s, and then gradually returned to the original level after 4 to 5 min. In *Chlamydomonas* we found that fast and repetitive $[Ca^{2+}]_{cyt}$ spikes were recorded after the perception of hypoosmotic shock. Moreover, similarly to what was seen in tobacco (Takahashi et al., 1997), extracellular Ca^{2+} has shown to be needed for intracellular $[Ca^{2+}]$ elevations in the green alga as no dynamics were recorded in absence of external free Ca^{2+}. An additional confirmation about the involvement of Ca^{2+} influx in the production of the observed multiple variations in

$[Ca^{2+}]_{cyt}$ was given by the use of mechanosensitive ion channels blockers. GsMTx4 and Ruthenium Red (RuR) has previously shown to partially reduce intraflagellar Ca^{2+} elevations in *Chlamydomonas* in response to flagella movement, with a higher effect for RuR than GsMTx4 (Collingridge et al., 2013). The effect or ruthenium red has to be carefully considered, as this inhibitor is not specific for MSCs. From our results, we can assume that stretch-activated channels in the cell body of *Chlamydomonas*, previously reported to be located on the plasma membrane and permeable to Ca^{2+} (Yoshimura, 1998), are involved in the observed Ca^{2+} dynamics. Indeed, GsMTx4 caused either a reduction in the number of cells that responded or a decrease in the number and magnitude of events in those that showed $[Ca^{2+}]$ elevations after the perception of the hypoosmotic stress. These results were confirmed by the total inhibition of $[Ca^{2+}]_{cyt}$ spikes caused by RuR in response to the same shock. Anyway, it is possible that the MSC channels may not be directly responsible for the observed Ca^{2+} dynamics. In fact, they might be non-selective for Ca^{2+}, but their activity might activate Ca^{2+} channels located on the plasma membrane.

Our results used two different hypoosmotic stresses (*i.e.* from CAB to distilled H_2O and from 100 mM sucrose CAB to distilled H_2O) to challenge *Chlamydomonas* cells. Switching from CAB to H_20 did not cause visible swelling (increase in cell volume), but there was an associated movement that may represent an osmotic adjustment of the cell. Both shocks caused repetitive Ca^{2+} elevations, although CVs were active at the time of the shock only in the first condition. Therefore, we propose that active CVs do not prevent hypoosmotic Ca^{2+} signalling and that a large increase in cell volume is not required to initiate the $[Ca^{2+}]$ elevations. Interestingly, the influx of water due to the stimulus caused swelling of cells, as reported in yeast (Nakayama et al., 2012) and *Fucus* (Goddard et al., 2000), and this increase in cell volume coincided with the multiple $[Ca^{2+}]_{cyt}$ oscillations recorded. Moreover, the swelling was also noticed in presence of the blockers, but Ca^{2+} dynamics were completely different than the control. This evidence strengthened our hypothesis for the involvement of mechanosensitive ion channels in the observed intracellular Ca^{2+} dynamics. A stretch-activated Ca^{2+} channel (MSC1) localized in the chloroplast of *Chlamydomonas* has been characterized from a molecular and electrophysiological point of view, but the presence of two other genes (*MSC2-3*) has been proved in this organism (Nakayama et al., 2007). It is then possible that one or either these two homologues encode for mechanosensitive Ca^{2+} channels localized on the plasma membrane. Interestingly, no Ca^{2+} dynamics and noticeable swelling were documented in wall-less strains, were the internal osmolarity was attested to be higher than the walled one

(Komsic-Buchmann et al., 2014). This evidence could point out an involvement of the cell wall in the perception of changes in external osmolarity, or that the stress is perceived differently due to these changes in cellular solute composition.

A distinct feature of *Chlamydomonas* compared to higher plants is the presence of one or two osmoregulatory compartments, the contractile vacuoles (CVs), which periodically accumulate and expel the excess of water. As the formation of CVs (diastole) involves the fusion of multiple small vesicles (Lukyx et al., 1997), and some intracellular membrane fusion processes require Ca^{2+} (Hay, 2007), we investigated for a role in CVs activity of the repetitive $[Ca^{2+}]_{cyt}$ elevations observed after the perception of the hypoosmotic shock in *Chlamydomonas*. The adaptation of cells to a mildly hyperosmotic environment allowed us to stop the activity of the vacuoles, and the subsequent shock with water exposed them to a significant change in external osmolarity. This treatment caused a visible swelling in nearly half of the cells but $[Ca^{2+}]_{cyt}$ increases were recorded only in less than 25% of the cases. Table 1 indicates that 18 cells showed swelling and 50% of these exhibited Ca^{2+} elevations. Anyway, in almost all cells that underwent to an increase in their volume, the initiation of CVs activity was observed later than the beginning of the swelling, even without any Ca^{2+} dynamic. This information lead us to conclude that the repetitive $[Ca^{2+}]_{cyt}$ elevations induced in response to hypoosmotic stress are not needed for the onset of intracellular vesicle fusion that then take to the formation of mature and functional CVs.

Future directions

As Ca^{2+} does not seem to be involved in the regulation of CVs, its role in the perception of a hypotonic environment should be investigated in other directions. The use of *Chlamydomonas* mutants with an impaired function of CVs activity that affects their osmoregulation (Luykx et al., 1997; Komsic-Buchmann et al., 2014) might clarify if Ca^{2+} is important in various cell processes. These functions could include exocytosis of vesicles to increase plasma membrane surface during swelling or the Ca^{2+}-dependent activation of K^{+} channels for the maintenance of the ionic balance to avoid cell burst. Moreover, *Chlamydomonas* mutants lacking mechanosensitive ion channels located in the plasma membrane could help to identify some of the mechanisms responsible for observed Ca^{2+} dynamics in response to the hypoosmotic shock. The hypothesis of an intracellular signalling role for Ca^{2+} in the freshwater alga has to be taken in account, especially if we considered that some studies reported the presence of different Ca^{2+} sensors. Indeed,

calmodulin (CaM) and CaM-like proteins has been assessed in *C. reinhardtii* (Zhu et al., 2015) as well as Ca^{2+}-dependent protein kinases (Hamel et al., 2014). On the other hand, no calcineurin B-like proteins (CBL) and CBL-interacting protein kinases coding genes have been found in its genome (Weinl & Kudla, 2009; Kleist et al., 2014). These differences between *Chlamydomonas* and other plants in the array of Ca^{2+} decoders, driven by selection favouring a complex system for processing information about the external environment, could have led to a diversification in the use of Ca^{2+} as a intracellular messenger. To go deeper in understanding Ca^{2+} handling in the green alga, targeting of genetically encoded Ca^{2+} indicators to different subcellular localizations, such as mitochondria or chloroplasts, would help to determine how different compartments are involved in Ca^{2+} homeostasis and signalling.

Concluding remarks

In this work the involvement of Ca^{2+} in the perception of some environmental stimuli in the freshwater alga *C. reinhardti* is discussed. From our results, it seems that some aspects of Ca^{2+} signalling do not appear to be conserved between *Chlamydomonas* and higher plants. On the other hand, we evidenced that osmotic responses might have some common features, at least in the involvement of Ca^{2+} in the perception of these stresses. Despite of some similarities, *Chlamydomonas* Ca^{2+} signals exhibited unique properties, *e.g.* fast Ca^{2+} waves and multiple rapid elevations. These differences may be due to the different Ca^{2+} signalling toolkits found in plants/algae and to their differing physiologies.

Ultimately, understanding differences in the way plants/algae respond to osmotic stress has important implications for clarifying how plants managed to colonize different terrestrial environments.

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CHAPTER 8.

Conclusions

The role covered by plant subcellular compartments in Ca^{2+} homeostasis and signalling is a rapidly developing and thrilling field of study (Stael et al., 2012). Attention has been mainly payed so far to the vacuole, which is the major Ca^{2+} storage compartments of the plant cell (Peiter, 2011). In the last few years, a growing interest has been focused on the involvement of chloroplasts, specific organelles that define plants, in the plant intracellular Ca^{2+}-regulated signalling network (Rocha & Vothknecht, 2012; Nomura & Shiina, 2014; Hochmal et al., 2015; Stael et al., 2015). Nevertheless, knowledge of the regulation of $[Ca^{2+}]$ inside chloroplasts is still limited; moreover, research studies have considered so far only chloroplasts, *i.e.* the photosynthetic functional type of plastids, whereas no information is available about other types of plastids.

This PhD thesis work aimed to uncover various aspects concerning the involvement of plastids in shaping subcellular Ca^{2+} dynamics triggered in response to environmental stimuli in *Arabidopsis thaliana*. Research purposes were also widened to the conservation of Ca^{2+} signalling mechanisms in the green lineage, by considering the ability of the freshwater unicellular green alga *Chlamydomonas reinhardtii* in evoking Ca^{2+} changes after the perception of external stresses.

The set-up of *Arabidopsis thaliana* heterotrophic and autotrophic cell suspension cultures allowed to dissect stimulus-specific Ca^{2+} signals in amyloplasts and chloroplasts. On the basis of the timing of the transient $[Ca^{2+}]$ elevations recorded in the cytosol, at the outer membrane of the plastid envelope and in the stroma in response to several abiotic stimuli, a role for plastids in switching-off cytosolic Ca^{2+} signals was proposed. Non-green plastids were found to be involved in Ca^{2+}-mediated signal transduction in a manner similar to photosynthetic ones. Nevertheless, significant differences in the amplitude of some stromal Ca^{2+} changes (*i.e.* those evoked by NaCl and mannitol) were found to be triggered between light-reactivated *versus* dark-adapted chloroplasts, suggesting a role for the photosynthetic status of the organelle in determining organellar Ca^{2+} signatures. Ca^{2+} signalling during drought and salinity stresses is highly integrated into many of the other signalling networks that regulate plant acclimation, including ROS, hormones and protein phosphorylation (Miller et al., 2010), and ROS are indeed produced in light-exposed chloroplasts.

Moreover, in this work the involvement of the thylakoid system in plastidial Ca^{2+} fluxes in response to environmental stimuli was also investigated. To address this issue, a new toolset of YFP-aequorin chimeras targeted to the thylakoid membrane and the thylakoid lumen was set up. Genetically encoded Ca^{2+} indicators have indeed been shown to be ideal probes to measure [Ca^{2+}] in intracellular compartments, because they can be modified to include specific targeting sequences (Rudolf et al., 2003). In particular, aequorin offers some advantages over GFP-based Ca^{2+} reporters for this type of studies, because it provides accurate [Ca^{2+}] measurements over a wide range of [Ca^{2+}], it has a high signal-to noise-ratio, and it is nearly insensitive to pH changes (Brini, 2008). These two latter properties make aequorin a suitable probe to sensitively and reliably measure [Ca^{2+}] in chloroplasts, although it does not allow subcellular imaging, because of low light emission (Martí et al., 2013). Monitoring of sub-chloroplast Ca^{2+} dynamics revealed the occurrence of stimulus-specific Ca^{2+} signals, characterised by unique kinetic parameters, and a role for the thylakoid lumen in the modulation of stromal Ca^{2+} signals was highlighted for the first time. In particular, evidence was provided for dark-stimulated intra-chloroplast Ca^{2+} fluxes, which suggested a new scenario for light-to-dark-induced Ca^{2+} transport inside the organelle. A pharmacological approach supported the likely involvement of an as yet unidentified thylakoid membrane-localized Ca^{2+}/H^{+} exchanger in the generation of the observed dark-induced luminal [Ca^{2+}] increase (Ettinger et al., 1999). To get insights into the homeostatic mechanisms allowing for chloroplast Ca^{2+} fluxes, a mutant line lacking a plastidial homologue of the mitochondrial calcium uniporter (MCU) was transformed with the construct encoding the stroma-targeted aequorin chimera. Ca^{2+} measurements assays in response to environmental stimuli indicated that this Ca^{2+} channel is involved in plastidial Ca^{2+} fluxes and it is possibly localized on thylakoid membranes. These results shed new light on the complex network underlying the Ca^{2+}-mediated transduction of environmental stimuli in plants at an intracellular level.

Ca^{2+} is well known to be used as an intracellular messenger by a wide variety of organisms, ranging from prokaryotes to eukaryotes (Dominguez, 2004; Case et al., 2007; Plattner & Verkhratsky, 2013), but research studies on the conservation of Ca^{2+} signalling mechanisms in the *Viridiplantae* clade, *i.e.* the green lineage, are still limited. In particular, it would be interesting, from an evolutionary point of view, to understand if the role of organelles in Ca^{2+} homeostasis and signalling is maintained among different photosynthetic organisms. As the chloroplast is their typical organelle, the conservation of

its role in shaping intracellular Ca^{2+} dynamics in green organisms of different taxonomic positions would undoubtedly be fascinating.

During the research period that I spent at the Marine Biological Association of the United Kingdom, the involvement of Ca^{2+} in the perception of environmental stimuli in the freshwater unicellular green alga *C. reinhardtii* was analysed. The lack of data regarding the monitoring of cytosolic [Ca^{2+}] ([Ca^{2+}]$_{cyt}$) and its changes during Ca^{2+}-mediated signal transduction in this microalga compelled to consider this aspect before investigating the role of the chloroplast. *Chlamydomonas* cells loaded with the fluorescent Ca^{2+}-sensitive Oregon green BAPTA were used to monitor [Ca^{2+}]$_{cyt}$ dynamics in response to the same environmental stimuli that are known to trigger transient [Ca^{2+}]$_{cyt}$ increases in *Arabidopsis*. No Ca^{2+} changes were recorded in response to mannitol and H_2O_2, but elevations in [Ca^{2+}]$_{cyt}$ were observed after the perception of NaCl and hypoosmotic stress. As a consequence, it can be speculated that some stimuli that give robust and reproducible responses in land plants do not appear to elicit Ca^{2+} signalling in *Chlamydomonas*. Moreover, the role of mechanosensitive ion channels in generating the observed Ca^{2+} dynamics in response to hypoosmotic stress was assessed. Differences in Ca^{2+} changes in response to external stimuli between higher plants and microalgae might be due either to the differential conservation of Ca^{2+} channels in cellular membranes (Wheeler & Brownlee, 2008; Verret et al., 2010; Edel & Kudla, 2015), or to the evolution of different systems to cope with changes in environmental conditions. These results might stimulate future research regarding the potential role of *Chlamydomonas* chloroplast in modulating intracellular Ca^{2+} dynamics.

Final remarks - The results obtained in this PhD work demonstrate the relevant involvement of plastids in Ca^{2+} fluxes activated in response to different environmental stimuli, as variations in [Ca^{2+}] were recorded at the surface of the organelle, in the stroma and in the thylakoid lumen. The different timing of [Ca^{2+}] variations observed in the various chloroplast sub-compartments indicate that these organelles may play a role in modulating intracellular [Ca^{2+}] through the dissipation of transient increases in [Ca^{2+}]$_{cyt}$. On the other hand, in particular conditions, such as after light-to-dark transition, Ca^{2+} responses in chloroplasts seem to take place independently of cytosolic [Ca^{2+}] dynamics. On the basis of these data, it may be hypothesized that chloroplast Ca^{2+} signals cover specific physiological roles in the organelle, regulating metabolic processes that take place in it. For example, dark-induced transient increases in stromal [Ca^{2+}] are believed to affect

the activity of some enzymes of the Calvin-Benson cycle, that are inhibited during the night (Sai & Johnson, 2002). Moreover, evidence for pathogen elicitor-triggered chloroplast Ca^{2+} signals in the activation of inter-organellar signalling has recently been demonstrated. Chloroplast Ca^{2+} signals were found to indirectly activate, possibly through a ROS-mediated retrograde signalling, the expression of nuclear-encoded genes and the consequent induction of defence responses in *Arabidopsis thaliana* (Nomura et al., 2012).

The different aequorin chimeras targeted to chloroplast sub-compartments turned out to be a valuable tool to the monitoring of organellar Ca^{2+} dynamics during signal transduction. This approach may blaze the trail for further investigations regarding the role of plastids in the complex Ca^{2+}-mediated signalling network of the plant cell.

For a wider view regarding this topic, future studies will have to consider the induction of responses downstream of the chloroplast-located Ca^{2+} signals, such as gene expression. Indeed, chloroplasts are semi-autonomous organelles, endowed with their own organellar genome. It can be envisaged that the induction of chloroplast Ca^{2+} signals may have direct effects on chloroplast gene expression, *i.e.* on the activation of potentially Ca^{2+}-regulated organellar genes. A currently fascinating research field regards the search for Ca^{2+} sensor proteins localized in chloroplasts, such as CaM and CaML or Ca^{2+}-dependent protein kinases, that could function as molecular decoders of organellar [Ca^{2+}] signals (Rocha & Vothknecht, 2012). Plastome gene expression analyses in response to the tested stimuli and the effective inhibition of the recorded [Ca^{2+}] variations through, for example, a pharmacological approach or the use of knock-out mutants, may unveil unexpected roles of organellar Ca^{2+} signalling. These studies may indeed uncover the potential Ca^{2+} dependency of the expression of some organellar genes, allowing to reveal new chloroplast-restricted responses.

From an evolutionary point of view, it is interesting to consider analogies in Ca^{2+} signalling between plastids and cyanobacteria, on the basis of the endosymbiotic origin of these organelles. Specific Ca^{2+} responses were recorded in *Anabaena* sp. induced by salinity, drought and cold shock (Torrecilla et al., 2000 and 2001). The fact that cyanobacteria are endowed with Ca^{2+}-based signal transduction mechanisms demonstrates an evolutionarily ancient origin of [Ca^{2+}] regulation in plastids.

In conclusion, this work provides some foundation stones to further investigate and study in deep the involvement and precise role of plastids in intracellular Ca^{2+} homeostasis and signalling in higher plants, together with novel hints to explore the ability of the green

unicellular alga *Chlamydomonas* to respond to and cope with changes in environmental conditions.

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