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Harnessing the new emerging imaging technologies to uncover the role of Ca^{2+} signaling in plant nutrient homeostasis

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Abstract

Increasing crop yields by using eco-friendly practices is of high priority to tackle problems regarding food security and malnutrition worldwide. A sustainable crop production requires a limited use of fertilizer as well as the employment of plant varieties with improved ability to acquire nutrients form soil. To reach these goals, the scientific community aims to understand plant nutrients homeostasis by deciphering the nutrient sensing and signaling mechanisms of plants. Several lines of evidence about the involvement of Ca^{2+} as the signal of an impaired nutrient availability have been reported. Ca^{2+} signaling is a tightly regulated process which requires specific protein toolkits to perceive external stimuli and to induce the specific responses in the plant needed to survive.

Here we summarize both older and recent findings concerning the involvement of Ca^{2+} signaling in the homeostasis of nutrients. In this review we present new emerging technologies, based on the use of genetically encoded Ca^{2+} sensors and advanced microscopy, that offer the chance to perform *in planta* analyses of Ca^{2+} dynamics at cellular resolution. The harnessing of these technologies with different genetic backgrounds and subjected to different nutritional stresses will provide important insights to the still little-known mechanisms of nutrient sensing in plants.

Summary statement

The role of Ca^{2+} signaling in plant sensing of both macro- and micronutrients has been firmly established. The same Ca^{2+}-decoding systems seems to be required for the integration of different signals. The use of modern imaging technologies to monitor *in vivo* Ca^{2+} dynamics will be of paramount importance for future researches in this important field.

Keywords

Introduction to plant mineral nutrition

Plants represent the main source of the essential mineral elements required by humans and other animals. The concentrations of mineral elements in edible plant tissues are thus of fundamental importance for the food chain (White & Brown, 2010). Furthermore, plants, residing in diverse and variable environments, require a steady, suitable balance of nutrients to maintain physiological processes (Rouached & Phan Tran, 2015). Indeed, in order to sustain growth and development plants must acquire an optimal amount of nutrients. However, the ability of plants to take up nutrients depends on the bioavailability of elements in the soils as well as on the health status of the plant itself. Variation in the physico-chemical properties of soil (such as pH, redox potential) decreases or increases the availability of some elements (White & Brown, 2010). Many environmentally stressful factors such as drought, high temperature and salinity affect plant growth by changing the nutrient status of plants. Conversely, the maintenance of an optimal nutrient status is crucial to sustain acclimation and adaptation responses of plants to stress conditions and thereby to allow plant growth and production.

It is known that limitations in crop yield productivity significantly contribute to the pressing problem of food security and malnutrition worldwide. To increase crop yields, fertilizers have been extensively used during recent decades. However, nowadays it is mandatory to identify new eco-friendly and sustainable practices for plant growth to limit the intensive use of fertilizers. To reach this goal, a better understanding of how plants perceive the external nutrient availability and how they regulate and integrate the homeostasis of different nutrients is of high priority.

As occurred for other cellular compound categories (such as transcripts, proteins and metabolites) whose profiles have been defined as transcriptome, proteome and metabolome, the mineral nutrients composition of an organism is often referred to as the ionome (Baxter, 2008, Coubert et al., 2019). Ionome includes all the mineral elements, which can be classified into the macronutrients (nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S)), and into the micronutrients (chlorine (Cl), boron (B), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni) and molybdenum (Mo)) (White & Brown, 2010; White et al., 2017). As stated before, to ensure plant requirements, the plant ionome profile results from a tight regulation of different processes such as uptake, translocation, storage and remobilisation of the nutrients (Lahner et al., 2003; Baxter et al., 2008; Baxter, 2015; Briat, Rouached, Tissot, Gaymard, & Dubos, 2015; Coubert et al., 2019). Since some

elements display chemical analogy or similarities among each other, the ionome composition of plants is determined by the multi-level interactions occurring among nutrients (Baxter et al., 2008). Such similarity can be observed for some elements at the transport level. For example, the IRT1 (IRON REGULATED TRANSPORTER 1) can take up Fe^{2+} as well as other divalent cations such as Mn^{2+}, Co^{2+}, Zn^{2+} and cadmium (Cd^{2+}) (Baxter et al., 2008).

Generally, as occurs under different environmental conditions, regulation of plant response to nutrient starvation occurs at different levels: 1) perception of external/internal nutrient status (nutrient sensing); 2) amplification of the perceived stimulus through transduction mechanisms (nutrient signaling); 3) activation of acclimation responses to the stress.

While acclimation responses to nutrient starvation represent the most investigated issue in the plant mineral nutrition field, the nutrient sensing and signaling topics remain to be fully elucidated. Nowadays, numerous insights about the molecular mechanisms regulating specific nutrient homeostasis have been identified (Kudoyarova, Dodd, Veselov, Rothwell, & Veselov, 2015; Rouached & Phan Tran, 2015). Nutrient sensing is a challenging issue in plants and some evidence has demonstrated the existence of specific receptors involved in the sensing mechanism for some nutrients. Significant progress has been made towards understanding the mechanism of how plants sense and respond to external N conditions. Evidence revealed that the nitrate (NO_3^-) transporter CHL1/NRT1.1/NPF6.3 located at the plasma-membrane also acts as a nitrate receptor (see below), thus defined as transceptor (Ho, Lin, Hu, & Tsay, 2009). Accordingly, SULTR1;2 and IRT1 transporters likely act as transceptors for S and Fe sensing respectively (Dubeaux, Neveu, Zelazny, & Vert, 2018; Zeng, Zhang, & Leustek, 2014).

The characterization of the transduction/signaling pathways of perceived nutrient imbalance in plants represents a challenging issue. As recently highlighted in Wilkins et al. (2016) Ca^{2+}-mediated signaling appears to play a central role in the regulation of nutrient deficiency stress. Here, the recent advances in this scientific area as well as the role of the new emerging imaging technologies for studying it will be discussed.

The role of Ca^{2+} as second messenger

Calcium ions (Ca^{2+}) are ubiquitous intracellular signaling messengers (Clapham, 2007). The origin of the signaling functions of Ca^{2+} likely resides in its chemical property, the ability to bind and precipitate phosphates, including ATP, a condition that cells must avoid (Clapham, 2007). For this reason, cells have evolved to invest much of their energy in maintaining cytosolic Ca^{2+} concentrations at low nM ranges (100-200 nM) which becomes crucial for Ca^{2+}-

based signaling (Clapham, 2007). In plants, rapid and transient increase of cytosolic Ca^{2+} concentration mediated by the opening of Ca^{2+}-permeable channels (e.g. GLRs, CNGCs, OSCAs, MCAs, Annexins and potentially TPC1) or by downregulation of Ca^{2+}-export systems (e.g. ACAs and ECAs Ca^{2+}-ATPases as well as CAXs) (Figure 1) (reviewed in Bose, Pottosin, Shabala, Palmgren, & Shabala, 2011; Kudla et al., 2018; Swarbreck, Colaço & Davies, 2013) allow transient increases of Ca^{2+} concentration that function as a versatile second messenger (Clapham, 2007; Sanders, Pelloux, Brownlee, & Harper, 2002; Kudla et al., 2018). In plant cells, different stimuli are associated with specific intracellular Ca^{2+} concentration changes characterized by given amplitudes and durations that might be decoded by different cellular Ca^{2+} sensors (see e.g. Evans, McAinsh, & Hetherington, 2001; Monshausen, 2012; Sanders, Pelloux, Brownlee, Harper, 2002; Trewavas, Read, Campbell, & Knight, 1996; Whalley & Knight, 2013; Xiong et al., 2006). Plants possess many proteins predicted to bind Ca^{2+} through EF domains (nearly 250 in Arabidopsis) (Day, Reddy, Shad, Ali, & Reddy, 2002; DeFalco, Bender, & Snedden, 2009). Calmodulin (CaM) (5 isoforms in Arabidopsis), Calmodulin like proteins (CML) (50 isoforms in Arabidopsis), Calcium-dependent protein kinases (CPK) (34 isoforms in Arabidopsis), Calcineurin B-like proteins (CBL) (10 isoforms in Arabidopsis) with their CBL-interacting protein kinase (CIPK) (26 members in Arabidopsis) partners represent the most studied classes of Ca^{2+} binding proteins that work as Ca^{2+} sensors/responders (reviewed in DeFalco Bender, & Snedden, 2009; Dodd, Kudla & Sanders, 2010; Edel, Marchadier, Brownlee, Kudla, & Hetherington, 2017; Kudla, Batistic, & Hashimoto, 2010; Kudla et al., 2018). Many plant scientists assign a role of Ca^{2+} in a signaling process based on the evidence that an applied stimulus, or a given growth condition, affects the resting cytosolic Ca^{2+} concentrations. In addition, forward and reverse genetic screenings that led to the identification of genes coding for Ca^{2+} binding proteins put Ca^{2+} into the signaling game.

Methods to measure Ca^{2+} dynamics *in vivo*

To hypothesize whether Ca^{2+} plays a role in plant nutritional signaling, the study of its *in vivo* intracellular concentration/homeostasis should be addressed. This is now possible thanks to molecular and imaging technologies. Analysis of Ca^{2+} dynamics in living plants was initially addressed by using Ca^{2+} sensitive dyes (e.g. Fura-2, Fura-2 dextran and Calcium Green Dextran) loaded in guard cells, pollen tubes and root hairs (Ehrhardt, Wais, & Long, 1996; Holdaway-Clarke, Feijo, Hackett, Kunkel, & Hepler, 1997; McAinsh, Webb, Taylor, &

Hetherington, 1995). The use of these dyes led to the gaining of insight into Ca^{2+}-signaling processes, but they presented some limitations (reviewed in Costa, Navazio, & Szabo, 2018). Hence, the introduction of the Genetically Encoded Calcium Indicators (GECI) (Knight, Campbell, Smith, & Trewavas, 1991; Miyawaki et al., 1997; Perez Koldenkova, & Nagai, 2013) was a sort of revolution that allowed a non-invasive monitoring of free Ca^{2+} levels, enabling real-time, spatially and temporally-resolved imaging of Ca^{2+} levels in different cell types and subcellular compartments (reviewed in Costa & Kudla, 2015; Costa, Navazio, & Szabo, 2018; Stael, Wurzinger, Mair, Mehlmer, Vothknecht, & Teige, 2012).

In plants, thus far the two mainly used GECIs have been aequorin and Cameleon (Costa & Kudla, 2015; Knight & Knight, 1995; Mithofer, & Mazars, 2002). The first GECI ever used was aequorin, which enabled monitoring of Ca^{2+} dynamics by photon emission measurements in transformed plants after reconstitution of the aequorin holoenzyme with the exogenously applied prosthetic group coelenterazine (Knight et al., 1991). Aequorin has represented and still represents a reliable tool to determine Ca^{2+} dynamics triggered by different stimuli particularly at the level of cell populations or entire plants, however it suffers from poor spatial resolution. Later, in the late 90s the use of the ratiometric Ca^{2+} reporter Cameleon based on combinations of spectral variants of the green fluorescent protein (e.g. CFP and YFP or cpVenus) has advanced the spatio-temporal resolution and sensitivity of Ca^{2+} signaling studies (Allen et al., 1999; Krebs et al., 2012; Miyawaki et al., 1997; Rudolf, Mongillo, Rizzuto, & Pozzan, 2003). Besides aequorin and Cameleon (particularly the YC3.6 version (Krebs et al., 2012; Nagai, Yamada, Tominaga, Ichikawa, & Miyawaki, 2004)) in recent years other GFP-based Ca^{2+} biosensors have been developed and successfully used in plants. We can cite for example the intensiometric Case12, GCaMP3, GCaMP6 and the green and red variant of GECO1 (G-Geco1 and R-Geco1) (Keinath et al., 2015; Kelner, Leitao, Chabaud, Charpentier, & de Carvalho-Niebel, 2018; Liu et al., 2017; Ngo, Vogler, Lituiev, Nestorova, & Grossniklaus, 2014; Vincent et al., 2017; Zhu et al., 2014). The difference between ratiometric and intensiometric sensors is based to their design. Some GECIs contain two fluorescent proteins (ratiometric sensors) and some contain a single fluorescent protein (non-ratiometric or intensiometric). The Cameleon sensor exploits the Förster Resonance Energy Transfer (FRET) property occurring between one fluorescent protein that acts as a donor (e.g. CFP), that transfers absorbed energy, to a second fluorescent protein, the acceptor (e.g. YFP or cpVenus) when excited. The efficiency of FRET in the Cameleon sensor depends on the donor-acceptor distance which is dependent by the Calmodulin (CaM)-M13 Ca^{2+}-dependent interaction. The

CaM-M13 module sensor is sandwiched between the two fluorophores (Miyawaki et al., 1997). Thus, with Cameleon the readout is the ratio between the acceptor and donor fluorescence emissions which reduces artefacts due to the expression level of the sensor or focus changes (Perez Koldenkova, & Nagai, 2013). Intensiometric GECIs relies on a change in the sensor quantum yield, measured as a change of the intensity of the emitted fluorescence which depends primarily by the amount of Ca^{2+} bound to the sensory domain of the probe which is still, in most of the cases, a CaM domain (Perez Koldenkova, & Nagai, 2013). However, a change of the expression level of the sensor could be misinterpreted as a change in free Ca^{2+} concentration (Perez Koldenkova, & Nagai, 2013). To overcome this limitation intensiometric GECIs can be expressed together with another fluorescent protein which fluorescence emission is used as reference, thus the sensor becomes ratiometric being its readout the ratio between the fluorescence emissions of the sensor and the reference protein. Dual excitation ratiometric MatryoshCaMP6s and R-GECO1-mTurquoise sensors have been recently generated and successfully expressed in Arabidopsis plants (Ast et al., 2017; Waadt, Krebs, Kudla, & Schumacher, 2017; Zhu et al., 2014). For the sake of clarity, we can state that intensiometric biosensors exhibit a higher signal change compared to a FRET-based sensor (e.g. Cameleon) in response to several stimuli (Keinath et al., 2015; Kleist et al., 2017; Vincent et al., 2017; Waadt, Krebs, Kudla, & Schumacher, 2017; Nguyen, Kurenda, Stolz, Chételat, & Farmer, 2018; Toyota et al., 2018). However, at the present time the Cameleon sensor, thanks to its strong FRET-based ratiometric properties has shown to be particularly suited for the quantification of Ca^{2+} levels in different genetic backgrounds (wild type vs mutants) (Behera et al., 2017; 2018; Costa et al., 2017; Corso, Doccula, de Melo, Costa, & Verbruggen, 2018; Shkolnik, Nuriel, Bonza, Costa, & Fromm, 2018) as well as different growth conditions within the same genotype (e.g. Behera et al., 2017; Matthus et al., 2019; Quiles-Pando et al., 2013; Tian, Zhang, Yang, Wang, & Zhang, 2016), so is thus probably more indicated for the study of Ca^{2+} dynamics/homeostasis in different nutritional conditions. Remarkably, Cameleon and some intensiometric sensors are also available for the analyses of Ca^{2+} dynamics in different intracellular compartments such as mitochondria, chloroplasts/plastids, endoplasmic reticulum, nucleus and peroxisomes (Table 1) (reviewed in Costa, Navazio, & Szabo, 2018).

In conclusion nowadays, we can choose among plant lines expressing different types of Ca^{2+} sensors, the choice of which can be made based on the specific experiment we want to carry out (see Table 1 and Costa, Navazio, & Szabo 2018).

Introduction of Ca^{2+} signaling in macronutrient nutrition

Recent results have identified Ca^{2+} signaling components in the sensing of nutrients availability. Here we summarize the recent findings related to K, N, P and Mg homeostasis.

K homeostasis. One of the first examples showing an involvement of Ca^{2+} in nutritional sensing was reported by two independent groups in 2006 which found that low K^+ availability in the growth medium activated the inward-rectifying Shaker-like channel subunit Arabidopsis K^+ transporter 1 (AKT1) through phosphorylation (Li, Kim, Cheong, Pandey, & Luan 2006; Xu et al., 2006). Both groups revealed that the phosphorylation was mediated by the CBL1-CIPK23 and/or CBL9-CIPK23 complexes. CBL1 and CBL9 are two plasma membrane localized members of the CBL family (Batistič, Waadt, Steinhorst, Held, & Kudla, 2010), whereas CIPK23 is a member of the CIPKs, localized in the cytoplasm and nucleus, in which expression is induced in low K^+ (Xu et al., 2006; Batistič et al., 2010). The CBL1 and CBL9 which can redundantly interact with CIPK23 are able to target the kinase to the plasma membrane and activate, through phosphorylation, AKT1 (Li et al., 2006; Xu et al., 2006). The involvement of CBLs-CIPK23 complexes in this pathway permitted the hypothesis that the AKT1 activation is dependent on a Ca^{2+}-based signaling. However, the authors of both works did not show the occurrence of any low K^+-induced cytosolic Ca^{2+} increase. However, 10 years later the group of Joerg Kudla, thanks to high resolution *in vivo* Ca^{2+} imaging based on the use of Arabidopsis seedlings expressing the Cameleon YC3.6 (see above) (Krebs et al., 2012; Nagai, Yamada, Tominaga, Ichikawa, & Miyawaki, 2004) was able to provide evidence that a quick drop in K^+ availability (from 5 to 0 mM) triggered a transient cytosolic Ca^{2+} increase in the post-meristematic stelar tissue of the Arabidopsis root elongation zone (Behera et al., 2017). Interestingly, in the same work the authors also demonstrated that prolonged K^+ starvation generated, still in root cells, a sustained cytosolic Ca^{2+} elevation (Behera et al., 2017). Besides these cited works it was later evident that different species (rice and wine grape) also share similar mechanisms of CBL-CIPK regulation of K^+ channel activities (e.g. Cuellar et al., 2010; Li et al., 2014). To make the story even more convincing, another work demonstrated that different CBLs (CBL1, CBL8, CBL9, and CBL10) through an interaction with CIPK23 activate the high affinity Arabidopsis K^+ transporter HAK5 (Ragel et al., 2015). The overall model predicts that K^+ starvation induces a cytosolic Ca^{2+} increase which activates CBLs-CIPK23 complexes that simultaneously increase the efficiency of root K^+ uptake by regulating the activity of both low and high affinity K^+ transporters (Behera et al., 2017; Li et al., 2006; Ragel et al., 2015; Xu et al., 2006). Besides a positive regulation of AKT1 activity mediated

by the CBLs-CIPK23 complexes, a CIPK-independent CBL10 negative regulation of AKT1 also exists (Ren et al., 2013). In addition to this, HAK5 interacts with the INTEGRIN LINKED KINASE1 that in turn is an interactor of the Ca^{2+} sensor CML9, thus suggesting another possible level of HAK5 regulation mediated by Ca^{2+}-based regulation (Brauer et al., 2016). Thus Ca^{2+}-based signaling can regulate the activity of K^+ transporters in several different ways. We might speculate that different Ca^{2+} signatures could activate different Ca^{2+} sensors and regulate differentially the same or different target/s (Kudla et al., 2018; Sanders, Pelloux, Brownlee, Harper, 2002; Webb, McAinsh, Taylor, & Hetherington, 1996). However, such a demonstration will require extensive research and an improvement of both spatial and temporal resolution analyses of Ca^{2+} dynamics in response to different K^+ availability.

AKT1 and HAK5 are not the only K^+ transport mechanisms involved in the K^+ nutrition regulated by Ca^{2+}-dependent processes. Other channels like AKT2 or transporters like Two Pore K (TPK) are post-transcriptionally regulated by Ca^{2+}. AKT2 is a potassium channel playing a key role in plant nutrition since it mediates K^+ entry into the phloem at the level of source organs (e.g. root cells) but it also works as a cation leaker in the phloem of sink tissues to facilitate the loading of sugar (Deeken et al., 2002; Gajdanowicz et al., 2011; Lacombe et al., 2000). An interesting aspect of AKT2 function is that its gating depends, similarly to AKT1, on its phosphorylation status but the responsible kinase(s) and the target residue(s) have not been identified yet (Dreyer, Michard, Lacombe, & Thibaud, 2001; Held et al., 2011). Nevertheless, AKT2 activity might be regulated by Ca^{2+} in its targeting to the plasma membrane. In fact, the AKT2 sorting from the endoplasmic reticulum is facilitated by the interaction with the CBL4-CIPK6 complex which through a phosphorylation-independent mechanism drives its targeting to the plasma membrane and thus functionality in K^+ uptake/release (Held et al., 2011). Moreover, plant K^+ nutrition does not rely only on K^+ uptake from soil, but it also depends on its storage and release from the vacuole which works as a reservoir of K^+ (Martinoia, Massonneau, & Frangne, 2000). K^+ release from vacuoles is, at least in part, mediated by the activity of Two-Pore K^+ (TPK) channels (Gobert, Isayenkov, Voelker, Czempinski, & Maathuis, 2007; Latz et al., 2007) the gating of which can be regulated by cytosolic Ca^{2+} and pH (Bihler et al., 2005; Czempinski, Zimmermann, Ehrhardt, & Müller-Röber, 1997). Specifically, a feature of TPK channels is that they possess an EF-hand pair which is a direct target of Ca^{2+}. Thus, cytosolic Ca^{2+} transients evoked by K^+ depletion can potentially also affect TPKs, of which the activity is linked to nutrient remobilization and K^+ homeostasis (Gobert, Isayenkov, Voelker, Czempinski, & Maathuis, 2007).

N homeostasis. Another important macronutrient for plants is nitrogen (N). Nitrogen is adsorbed from the soil in two forms, as nitrate (NO_3^-) or ammonium (NH_4^+) ions with a preference to NO_3^-, since NH_4^+ is toxic at high mM concentrations (Xu, Fan, & Miller, 2012). Experimental evidence demonstrates that the transport mechanisms for both N forms are finely regulated by Ca^{2+}-mediated signaling, since in Arabidopsis the CBL1/9-CIPK23 complex can phosphorylate both the CHL1/NRT1.1/NPF6.3 NO3- transceptor and the AMT1;1 NH_4^+ transporter (Ho, Lin, Hu, & Tsay, 2009; Straub, Ludewig, & Neuhäuser, 2017). Similarly to what was discovered for the AKT1-mediated K^+ uptake, the NO_3^- transport across the plasma membrane through CHL1/NRT1.1/NPF6.3 is finely regulated by the CBL-CIPK complex which shifts its activity from a low- to a high-affinity transporter depending on the external availability of the ion (Ho, Lin, Hu, & Tsay, 2009).

N homeostasis: NO_3^-. Using Arabidopsis plants expressing aequorin it was demonstrated that plants grown in the presence of NH_4^+ as the sole N source and then exposed to mM concentrations of NO_3^- showed an immediate and transient cytosolic Ca^{2+} increase which requires the activity of the phospholipase C enzyme (Riveras, Alvarez, Vidal, Oses, Vega, & Gutiérrez, 2015). Such NO_3^--induced cytosolic Ca^{2+} increase may be ultimately responsible for the activation of the CBL-CIPK Ca^{2+} sensor and therefore the CHL1/NRT1.1/NPF6.3 regulation (Riveras, Alvarez, Vidal, Oses, Vega, & Gutiérrez, 2015). Following a different approach, the laboratory of Jen Sheen using the ultrasensitive Ca^{2+} sensor CGaMP6s (Chen et al., 2013) confirmed the existence of a NO_3^--induced Ca^{2+} increase in Arabidopsis mesophyll protoplasts and seedlings root tip cells (Liu et al., 2017). In addition, it was also revealed that the CPK10, CPK30 and CPK32 kinases act as master regulators of the primary NO_3^- response via the phosphorylation of the NIN-LIKE PROTEIN (NLP) transcription factor. Then the active form of NLP reprograms the expression of other downstream transcription factors as well as genes involved in nitrogen assimilation, carbon/nitrogen metabolism, redox signaling, hormones and proliferation (Liu et al., 2017). Overall a model can be predicted in which NO_3^- induces a cytosolic Ca^{2+} transient that regulates its own transport through a CBL1/9-CIPK23 mediated-mechanism and the gene transcription through CPK10, CPK30 and CPK32 (Ho, Lin, Hu, & Tsay, 2009; Liu et al., 2017). To make the scenario even more complex there is also the demonstration that among the NO_3^--regulated genes there are also those coding for the CIPK23 and CIPK8 kinases, the expression of which is downregulated through the activity of FACTOR INTERACTING WITH POLY(A) POLYMARASE 1 (FIP1) transcription factor (Wang et al.,

2018). Moreover, CIPK8 also regulates post-transcriptionally the activity of CHL1/NRT1.1/NPF6.3 NO_3^- transporter (Ho, Lin, Hu, & Tsay, 2009).

The CHL1/NRT1.1/NPF6.3, beside its direct role in transporting NO_3^- in a dual-affinity way depending on its phosphorylation status, can also transport auxin and it has recently been shown to work as a nutrient molecular integrator by controlling the phosphate (Pi) starvation response (Gojon, Krouk, Perrine-Walker, & Laugier, 2011; Medici et al., 2019). The CHL1/NRT1.1/NPF6.3 control of Pi starvation is mediated through the accumulation and turnover of the transcription factor PHOSPHATE STARVATION RESPONSE 1 (PHR1) which is itself a master regulator of Pi sensing and signaling (Medici et al., 2019). Pi is a macronutrient, deficiency of which strongly limits plant growth, and the plant roots need to adapt to optimize Pi uptake (Matthus et al., 2019). Pi depletion/starvation does not seem to induce cytosolic Ca^{2+} increase and thus potential Ca^{2+} sensors activation, but it has been shown to affect Ca^{2+}-signatures triggered by different external stimuli (Matthus et al., 2019). It was in fact demonstrated that Arabidopsis seedlings, grown under chronic Pi deprivation, showed altered root cytosolic Ca^{2+} responses to extracellular ATP, sorbitol and NaCl compared to control (Matthus et al., 2019). Remarkably, this change in stimulus-induced cytosolic Ca^{2+} dynamics was dependent on Fe accumulation in Pi starved plants (Matthus et al., 2019). These pieces of evidence reveal how plants might integrate nutritional and environmental cues, adding another layer of complexity to the use of Ca^{2+} as a signal transducer.

N homeostasis: NH_4^+. The NH_4^+ form is transported into the cells by high-affinity NH_4^+ transporters (AMTs) (Xu, Fan, & Miller, 2012) and to avoid toxic accumulation of cytoplasmic NH_4^+ the AMT transporter activity must be finely regulated. In *Arabidopsis thaliana* AMTs (AMT1;1 and AMT1;2) are efficiently inactivated by phosphorylation (Straub, Ludewig, & Neuhäuser, 2017) and surprisingly the kinase responsible for such inactivation is the CIPK23 in complex with CBL1 (Straub, Ludewig, & Neuhäuser, 2017). Interestingly, NH_4Cl treatment was shown to induce cytosolic Ca^{2+} increases in Arabidopsis roots using aequorin expressing plants (Plieth, Sattelmacher, & Knight, 2000). At present, we do not know exactly which mechanisms are activated in the NH_4^+-induced cytosolic Ca^{2+} increase but we might tentatively speculate that since external NH_4^+ can inhibit the high-affinity K^+ transport (Hirsch, Lewis, Spalding, & Sussman, 1998) the NH_4^+-induced cytosolic Ca^{2+} increase might be mediated by K^+-deprivation (Behera et al., 2017).

In summary, the transport of K^+, NO_3^- and NH_4^+ is highly regulated and interconnected, and this connection is possibly dependent on Ca^{2+} signalling, mainly through the activity of CBL1/9-CIPK23 complexes.

Mg homeostasis. A further macronutrient which is linked to Ca^{2+} homeostasis and signaling is magnesium (Mg^{2+}). Mg^{2+} is an essential macronutrient for plants, since it serves as a counter ion for nucleotides, it is a central metal for chlorophylls and it contributes to membrane stabilization and active conformation of macromolecules (Shaul, 2002). Both low and high levels of Mg^{2+} present in the soil are deleterious to plant growth, thus affecting crop production. Members of the Mg^{2+} transporters (MGTs) family have been identified to facilitate Mg^{2+} transport in Arabidopsis (Li, Tutone, Drummond, Gardner, & Luan, 2001). In yeast, Mg^{2+} deprivation elicits rapid Ca^{2+} influx and activates a Ca^{2+}/calcineurin signaling pathway (Wiesenberger et al., 2007). However, a similar mechanism has not been identified in plants. As detrimental effects on plant growth have been observed under high concentrations of environmental Mg^{2+} (Visscher et al., 2010) some studies established a critical function of Ca^{2+} signaling in plant adaptation to high Mg^{2+} stress (Tang et al., 2015). Two tonoplast-CBL proteins, CBL2 and CBL3, are predicted to perceive a transient rise of Ca^{2+} in the cytosol under Mg^{2+} excess conditions. After a putative Ca^{2+} signal, CBL2 and CBL3 activate a subset of CIPKs, such as CIPK3, 9, 23 and 26, which in turn phosphorylate the Mg^{2+} transport systems of the tonoplast, allowing vacuolar Mg^{2+} sequestration and avoiding the accumulation of toxic levels of Mg^{2+} in the cytosol (Tang et al., 2015). So far, the Mg^{2+} transporters localized at the tonoplast involved in such a mechanism are still unknown, as the AtMXT, a Mg^{2+}/H^+ antiporter (Amalou, Gibrat, Brugidou, Trouslot, & d'Auzac, 1992) as well as the MGT2 and MGT3 transporters, tonoplast-localised Mg^{2+} transporters (Conn et al., 2011) have been not involved in the Ca^{2+}-mediated vacuolar sequestration of Mg^{2+} under excess conditions (Tang et al., 2015).

Ca^{2+} signaling in micronutrient homeostasis

Ca^{2+}-mediated signaling has also been involved in the regulation of plant responses to micronutrient starvation. Among micronutrients, information collected concerns mainly Fe and B.

Fe homeostasis. In dicot plants the Fe uptake mechanism is based on a reduction-based strategy, where the ferric form (Fe^{3+}) of Fe is reduced at the root surface and imported as ferrous

Fe (Fe^{2+}) (Brumbarova, Bauer, & Ivanov, 2015). Fe starvation induces the expression of specific genes to control Fe homeostasis (Mai, Pateyron, & Bauer, 2016). The regulation of Fe acquisition mechanism in plants is mediated mainly by the basic helix-loop-helix (bHLH) FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT). FIT upregulates the genes encoding the FERRIC REDUCTASE-OXIDASE2 (FRO2) and the metal transporter IRON-REGULATED TRANSPORTER1 (IRT1) (Colangelo & Guerinot, 2004). Tian, Zhang, Yang, Wang, & Zhang (2016) found that Arabidopsis seedlings grown for four days in Fe deficiency conditions showed an increase of cytosolic Ca^{2+} in root cells, visualized in Arabidopsis plants expressing the Cameleon YC3.6. This result was later confirmed by Gratz et al. (2019) which reported an Fe-deficiency-dependent cytosolic Ca^{2+} increase in the early root differentiation zone, both in the central cylinder and in the epidermis. Remarkably, Tian, Zhang, Yang, Wang, & Zhang (2016) also demonstrated that the CBL1/9 CIPK23 complex was required for the modulation of iron uptake, possibly through a regulation of the ferric-chelate reductase activity. We can therefore speculate that the observed Fe-deficiency-induced cytosolic Ca^{2+} increase might be required for the activation of the CBL1/9-CIPK23 complex. Recently, it has been discovered that CIPK23 phosphorylates a histidine-rich stretch of IRT1 after direct metal binding. Phosphorylated IRT1 is then targeted to efficient endosomal sorting and vacuolar degradation (Dubeaux et al., 2018). Furthermore, it has been demonstrated that the ENHANCED BENDING (EHB1) protein acts as a direct inhibitor of IRT1 and that the EHB1-IRT1 interaction is promoted by Ca^{2+} (Khan et al., 2019). Further evidence of a link between Ca^{2+} signaling and Fe deficiency has been recently provided by Gratz et al. (2019), who demonstrated in Arabidopsis that the pool of functionally active FIT depends on phosphorylation of FIT protein in order to alter the plant's Fe utilization capacity. Such phosphorylation, occurring at Ser272, is mediated by the CIPK11 (Gratz et al., 2019) and discriminates between a small pool of active FIT (Ser272-phosphorylated) and a large pool of inactive FIT (non-Ser272-phosphorylated). Phosphorylation of FIT drives higher nuclear accumulation of FIT itself. In the nucleus, FIT can form homo-dimers and interact with bHLH039, a further Fe-deficiency-regulating transcription factor. Notably, the authors demonstrated that a defective mutant in *cipk11* affected FIT - bHLH039 interaction, leading to decreased FIT target gene induction under Fe deficiency. By contrast, enhanced Ser272-phosphorylated FIT-bHLH039 interaction activates FIT transcriptional activity, resulting in a higher level of FIT target gene expression. Interestingly, Fe availability affects the capacity of plants to respond to abiotic stress by altering the link between Reactive Oxygen Species (ROS) and Ca^{2+} in Pi-starved plants. Overall, it is known that different abiotic stresses determine ROS

accumulation in roots, mainly generated by NADPH oxidases (Demidchik, Shabala, & Davies, 2007; Wang et al., 2017). The induction of NADPH oxidases seems to be responsible of the activation of Ca^{2+} influx across the plasma membrane which, in turn, may lead to a cytosolic Ca^{2+} increase through the activation of ROS-activated ion channels (Laohavisit et al., 2012; Demidchik, 2018; Matthus et al., 2019). However, the ROS production in Pi-deprived plants is linked to the Fe accumulation occurring under Pi starvation (Balzergue et al., 2017; Müller et al., 2015). Indeed, the concomitant starvation of Pi and Fe restore the normal ROS level in the cell as well as the Ca^{2+} signature after exogenous application of ATP in Pi-starved root tips (Matthus et al., 2019). All these findings indicated that i) Ca^{2+} signaling is directly involved in the transduction of Fe deficiency signals and thereby in the activation of stress responses, and that ii) impaired Fe content might affect the ROS/ Ca^{2+} hub in signaling processes under Pi starvation.

B homeostasis. Upon B deprivation an increase of Ca^{2+} uptake has been observed in tobacco BY-2 cells expressing aequorin (Koshiba, Ishihara, & Matoh, 2010) as well as in Arabidopsis plants expressing Cameleon YC3.6 (Quiles-Pando et al., 2013). The expression of some genes encoding for Ca^{2+}-signaling proteins involved in Ca^{2+} transport, sensing and decoding was upregulated (Quiles-Pando et al., 2013). The involvement of Ca^{2+} permeable channels, responsible for the rise of Ca^{2+} concentration in the cytosol under B starvation, was hypothesized by the fact that lanthanum, a non-selective blocker of cation channels and stretch-activated Ca^{2+}-permeable channels (reviewed in De Vriese, Costa, Beeckman, & Vanneste, 2018) inhibited the cytosolic Ca^{2+} increase as well as an upregulation of the genes coding for the CNGC19 (a putative Ca^{2+}-permeable channel). Furthermore, the sensitivity of Ca^{2+} transport to diphenyleneiodonium (DPI) suggested an involvement of NADPH oxidase in the modulation of responses under B deficiency (Koshiba, Ishihara, & Matoh, 2010). In the same work the authors also demonstrated that B deficiency induced the expression of genes coding for three auto-inhibited Ca^{2+}-ATPases ACA1, ACA10 and ACA13 as well as the cation-H^+ antiporter (CAX3). In particular ACAs (ACA10 and ACA13) and CAX3 are supposed to withdraw Ca^{2+} into the apoplast and vacuole respectively to possibly re-establish the cytosolic Ca^{2+} resting after a transient increase. Interestingly, the increased Ca^{2+} level in the B-deprived cells might be linked to the increase of cyclic nucleotides in the cytosol resulting in the stimulation of CNGC activity (e.g. CNGC19) (Ma & Berkowitz, 2011; Yuen & Christopher, 2013) which favor a Ca^{2+} entry into the cytosol. Cyclic nucleotides could have a main role in

the timing of events triggered by B deficiency, as occurs in other stresses (Ma & Berkowitz, 2011; Yuen & Christopher, 2013), but this needs to be further investigated.

Genes encoding for CMLs (CML12, CML24, CML45, CML47) and for Ca^{2+}-dependent protein kinases (CPK28, CPK29), involved in Ca^{2+} sensing and decoding process respectively, were also up-regulated under B deprivation (Quiles-Pando et al., 2013). The role of such proteins in the modulation of B deficiency-induced responses has been attributed to the activation of the specific transcription factors (TFs), such those belonging to WRKY and MYB TFs families (González-Fontes et al., 2016; Wilkins, Matthus, Swarbreck, & Davies, 2016). Indeed, an upregulation of some WRKY, MYB TFs was observed under B deficiency (González-Fontes et al., 2016)

New molecular and imaging technologies to test new hypotheses in Ca^{2+}-based plant nutrition signaling

As mentioned above the study of *in vivo* Ca^{2+} dynamics/homeostasis in living plants, cultivated in different growth conditions, was made possible thanks to modern microscopy technologies. Whereas in its infancy *in vivo* Ca^{2+} imaging was mainly carried out in stomata guard cells and pollen tubes, in the last 10 years the technique was also established in root cells, becoming a routine in different laboratories (Behera & Kudla; 2013; Behera et al., 2017; Candeo, Doccula, Valentini, Bassi, & Costa, 2017; Costa, Candeo, Fieramonti, Valentini, & Bassi, 2013; Costa et al., 2017; Keinath et al., 2015; Kelner, Leitao, Chabaud, Charpentier, & de Carvalho-Niebel, 2018; Krebs et al, 2012; Krebs & Schumacher, 2013; Loro et al., 2012; Monshausen, Bibikova, Weisenseel, & Gilroy, 2009; Rincón-Zachary et al., 2010; Shih, DePew, Miller, & Monshausen, 2015; Shih, Miller, Dai, Spalding & Monshausen, 2014; Tanaka, Swanson, Gilroy & Stacey, 2010; Waadt, Krebs, Kudla, & Schumacher, 2017). The possibility of pursuing Ca^{2+} imaging analyses in root cells represents an important tool to understand the role of Ca^{2+} signaling in nutritional studies and high-resolution microscopy (e.g. wide field fluorescence microscopy (WF) and confocal microscopy) can also provide information about the tissue specificity of the responses which can help with the identification of the specific nutrient sensor (Table 2). For example, K^+ and Fe deprivation triggered a cytosolic Ca^{2+} increase in the post-meristematic stelar tissue of the Arabidopsis root elongation zone (Behera et al., 2017; Tian, Zhang, Yang, Wang, & Zhang, 2016; Gratz et al., 2019). B starvation and NO_3^- administration affected the Ca^{2+} levels in the root tip meristematic cells (Liu et al., 2017; Quiles-Pando et al., 2013). Pi deprivation altered the Ca^{2+} dynamics in the root transition zone,

but not in the root tip meristematic cells (Matthus et al., 2019). These results allow us to hypothesize that different tissues might show different sensitivities towards the various ions' availability. However, it must be said that the experiments here cited did not precisely compare the Ca^{2+} levels in different cell types and they did not evaluate in detail the existence of different tissues' sensitivities (only with Fe deprivation in Gratz et al., 2019). We might therefore be interested to answer a simple question: which is/are the root tissue/s that primarily sense/s a given nutrient deprivation and that activate Ca^{2+}-signaling responses? To answer this question, we need to carry out analyses at high spatial and temporal resolution. The generation of new transgenic lines expressing GFP-based sensors under the control of tissue specific promoters (e.g. epidermis, root hairs, endodermis, cortex etc.) would be useful. This strategy has been recently explored by generating plants expressing the GCaMP3 sensor (Tian et al., 2009) under the control of the SUC2 promoter which allowed the monitoring of the Ca^{2+} dynamics in the phloem of intact Arabidopsis leaves (Vincent et al., 2017; Toyota et al., 2018). Besides the study of the tissue specificity of nutrient-induced cytosolic Ca^{2+} responses, another important consideration is the design of a more physiological experimental approach. In fact, most of the Ca^{2+} imaging experiments performed with plants subjected to nutrient stress have been carried out using plants grown vertically on plates and then transferred to a horizontally laid microscope slide at different time points from the beginning of the treatment. Alternatively, the stimulus was applied by a direct administration of a solution containing the desired ion (Behera et al., 2017; Liu et al., 2017) in seedlings lying horizontally. Even if these approaches are reliable and have provided consistent data, they might not fully guarantee the maintenance of strict physiological conditions and can introduce artefacts due to sample manipulation (Candeo, Doccula, Valentini, Bassi, & Costa, 2017; Romano-Armada et al., 2019). Moreover, an experiment in which only a few time points are assayed might not guarantee catching the precise time at which a change in Ca^{2+} concentration occurs after, for example, a nutrient shortage. An important consideration is that a quick nutrient deprivation is a condition hardly seen in nature. It is more plausible that nutrient deprivation can occur with a slow washout which might bring about a progressive nutrient depletion and thus a progressive sensing that cannot induce a sharp increase of cytosolic Ca^{2+}. Thus, it would be significant to carry out experiments in which the monitoring of Ca^{2+} levels are performed in more physiological conditions in which a slow decrease or increase of nutrients is applied while a continuous imaging is carried out. An important breakthrough in this direction was reached by using a vertically positioned laser scanning confocal microscope (LSCM) which allowed scientists to perform a live tracking of vertically grown Arabidopsis seedlings roots (von Wangenheim et

al., 2017), or the use of the root chip microfluidics which allows a long-term imaging of different plant roots with a continuous perfusion of the medium that can be conveniently exchanged (Grossmann et al., 2011; Stanley et al., 2018). Another approach is represented by an imaging technology called Light Sheet Fluorescence Microscopy (LSFM) (Figure 2a) (reviewed in Berthet & Maizel, 2016; Ovečka et al., 2018). This technique allows the rapid acquisition of images with a high dynamic range with single cell resolution over a wide field of view and can be easily tailored to a given specimen, including vertically growing seedlings (Figure 2a,b) (Berthet & Maizel 2016; Costa, Candeo, Fieramonti, Valentini, & Bassi., 2013; Maizel, von Wangenheim, Federici, Haseloff, & Stelzer, 2011). One of the advantages of LSFM is the low dose of light needed to image, which drastically reduces phototoxicity, an important parameter to be considered in the case of long-term imaging experiments (Candeo, Doccula, Valentini, Bassi, & Costa, 2017; Ovečka et al., 2018). Besides the advantages due to the low phototoxicity, the rapid image acquisition enables the performance of experiments in which, at any given time point, a z-stack of the specimen can be acquired with the possibility of generating 4D reconstruction (Figure 2c). Remarkably, the fact that every single image can be stored and analysed off line can allow the acquisition of precise tissue specific information. Maizel, von Wangenheim, Federici, Haseloff, & Stelzer (2011) was the first group which exploited the use of LSFM in plants, demonstrating the real possibility of following Arabidopsis root growth in vertically maintained seedlings over several hours with cellular and subcellular resolution (Maizel, von Wangenheim, Federici, Haseloff, & Stelzer 2011). A few years later we implemented the LSFM technique for the analysis of stimulus-induced Ca^{2+} dynamics (Costa, Candeo, Fieramonti, Valentini, & Bassi 2013). In this latter case Arabidopsis plants expressing the Cameleon sensor YC3.6 localized in the cytosol and nucleus (Krebs et al. 2012) were employed and we showed that single cell Ca^{2+} dynamic analyses were possible in untouched, vertically grown roots (Costa, Candeo, Fieramonti, Valentini, & Bassi, 2013). Later on we carried out analyses of root hair tip Ca^{2+} oscillations in plants grown in standard growth medium or supplemented with 50 nM of auxin (NAA) (Candeo, Doccula, Valentini, Bassi, & Costa, 2017). Based on these new possibilities, it will be therefore desirable to design new experiments in which plants expressing a Ca^{2+} sensor can be grown in standard conditions and then subjected to a change in the medium composition while imaging, or to pursue long-term imaging where plants are grown in media with different compositions. The high spatial and temporal resolution offered by LSFM could also lead to the possibility of collecting images of entire volumes inside the roots (Figure 2c), thus allowing the making of comparisons among different tissues by measuring the levels of Ca^{2+}, but also the analysing of the morphology and

quantifying the size of the cells. Additionally, LSFM would help to explore how nutrient deprivation affects Ca^{2+} levels in subcellular compartments. In fact, the availability of transgenic lines expressing Ca^{2+} biosensors in mitochondria, plastids, endoplasmic reticulum etc (reviewed in Costa, Navazio, & Szabo, 2018, Table 1) can represent a new area of investigation. It must be said that LSFM, as every technology, displays limitations. LSFM spatial resolution is lower compared to a LSCM (Ovečka et al., 2018) and its use is particularly suited for imaging analyses in transparent low scattering tissues but limited in densely packed cells or green tissues (Ovečka et al., 2018). Thus, Ca^{2+} imaging analyses in green tissues can be better afforded by WF microscopy or, in case optical sectioning is needed, by LSCM or spinning disk confocal (SD) (Ovečka et al., 2018). As a final assessment we can consider that every microscopy technique has advantages and disadvantages (summarized in Table 2) and that they cannot strictly guarantee physiological conditions. We envisage that for long-term Ca^{2+} imaging LSFM could offer several advantages particularly for root tissues. However, also in this latter case the roots must be grown in a transparent soil or in solution (Maizel et al., 2011; Candeo, Doccula, Valentini, Bassi, & Costa, 2017) and the cells must be illuminated with a laser, a condition which is not found in nature and that can potentially have some effects on cell physiology.

Future perspectives in the signaling studies of plant nutrient homeostasis

Despite what we have summarized in this review, our feeling is that we have probably only scratched the surface about the study of the Ca^{2+} signaling role in the nutrient sensing mechanism of plants. In such a scenario we can better focus our attention on the study of the key components involved in any Ca^{2+}-based signaling process in response to nutritional stress. The basic Ca^{2+}-signaling components can be considered the following: i) Ca^{2+} influx system (responsible for the increase of Ca^{2+} concentration in the cytosol after stimulus sensing); ii) Ca^{2+}-decoding toolkit (responsible for the Ca^{2+}-mediated signal transduction) and iii) Ca^{2+} efflux systems (responsible for the restoration of Ca^{2+} concentration in the cytosol) (Figure 1) (Kudla et al., 2018). In the plant nutrition field, most of the information available is only related to the proteins involved in the Ca^{2+} decoding process, while only very little evidence is available about the specific Ca^{2+} influx and efflux systems involved in the generation and regulation of cytosolic Ca^{2+} increases (Figure 1). To fill this gap, the study of Ca^{2+} signaling in response to nutritional stress, in different genetic backgrounds of known mutants for Ca^{2+}

export systems (e.g. Ca^{2+}-ATPase or CAXs) or putative Ca^{2+} permeable channels, might provide useful clues. Besides, forward genetic screenings might be pursued through the mutagenesis of plant lines expressing Ca^{2+} sensors, scoring for plants showing altered Ca^{2+} homeostasis in given nutritional conditions. Thus, pursuing a Ca^{2+}-sensor-based screening in nutrient stress conditions might allow us to identify new signaling components possibly related to Ca^{2+} transport.

An interesting observation which raises fundamental questions is that except for B, altered homeostasis of different nutrients require the activity of CBL-CIPKs complexes (especially CIPK23) as the Ca^{2+} decoding system. As we stated before, interaction among nutrients represents an important cellular feature determining the plant ionome. Such interaction is mirrored also at the molecular level. For example, it has been demonstrated that the PHR1 is the first molecular link common to various pathways controlling mineral nutrition of both macro- and micro-elements (Briat Rouached, Tissot, Gaymard, & Dubos, 2015 and references therein). Therefore, we might suggest that different nutrient stresses can engage similar components of the Ca^{2+} signaling process, depending on the existing cross-talk occurring among the nutrients themselves. However, such observations raise the question of how the transduction signaling might be specific for a given nutrient. Overall the Ca^{2+} signaling specificity is still under debate in different biological fields. Both in plant and in animal cells it has been suggested that specific cell metabolic changes, mediated by Ca^{2+}, might be related to several features of the Ca^{2+} signal itself, like its magnitude, duration, localization and sustained/oscillatory nature which can potentially activate different Ca^{2+} sensors (Sanders, Pelloux, Brownlee, & Harper, 2002; Kudla, Batistic, & Hashimoto, 2010; Burgoyne & Haynes 2015). Although the responses to a given nutrient deficiency might results from an integration of a general stress related with specific stress-related signals, the role of Ca^{2+} in the modulation of nutrients homeostasis should be deeply investigated. We believe that new technologies will help us to better study the link between nutrient cross-talk and Ca^{2+} signaling by increasing the analytical sensitivity to monitor the above-mentioned features of Ca^{2+} signal as well as the spatial and temporal resolution of the analysis (Figure 1). Having the possibility to follow the cellular activities for prolonged time (e.g. by means of vertical confocal or LSFM) (Figure 2) of plants growing in different nutrient conditions with high spatial resolution can provide a map of the plant sites primarily involved in the sensing and activation of Ca^{2+}-mediated responses, as well as a precise temporal progression of signal transduction within a plant. Moreover, we might also consider pursuing analysis of Ca^{2+} dynamics at the subcellular level

(e.g. by using plant lines expressing Ca^{2+} sensors in different compartments), which will provide a new level of information. Cellular organelles (such as chloroplasts/plastids and mitochondria) are important targets of nutrient starvation, since i) they require several nutrients to keep metabolic processes working, and ii) they are engaged in the metabolic modulation required to sustain energetically the cellular responses to the nutrient stress (Vigani & Hanikenne, 2018). Recent works have identified transporters involved in the accumulation of Ca^{2+} in the mitochondrial matrix (Teardo et al., 2017) or in plastidial stroma (Frank et al., 2018) thus mutants for these transporters might be studied to understand whether they are involved in the regulation of cellular Ca^{2+} homeostasis. The fact that several indications show that organelles also play a role in the regulation of nuclear genes expression under nutrient starvation (Vigani, Zocchi, Bashir, Philippar, & Briat, 2013; Vigani et al., 2016; Vigani, Solti, Thomine, & Philippar, 2019) can represent another interesting aspect to investigate. We can hypothesize that the mechanism through which organelles communicate with the nucleus in nutritional sensing is by a proper control of organellar Ca^{2+} homeostasis. The combination of the use of new imaging technologies with Ca^{2+} sensors expressed in different genetic backgrounds, in different tissues and subjected to different nutritional stresses will provide the ground for new hypotheses and new tests.

Author contributions

G.V. and A.C. carried out the literature study, made the figures and wrote the manuscript.

Competing interests

The authors have declared that no competing interests exist.

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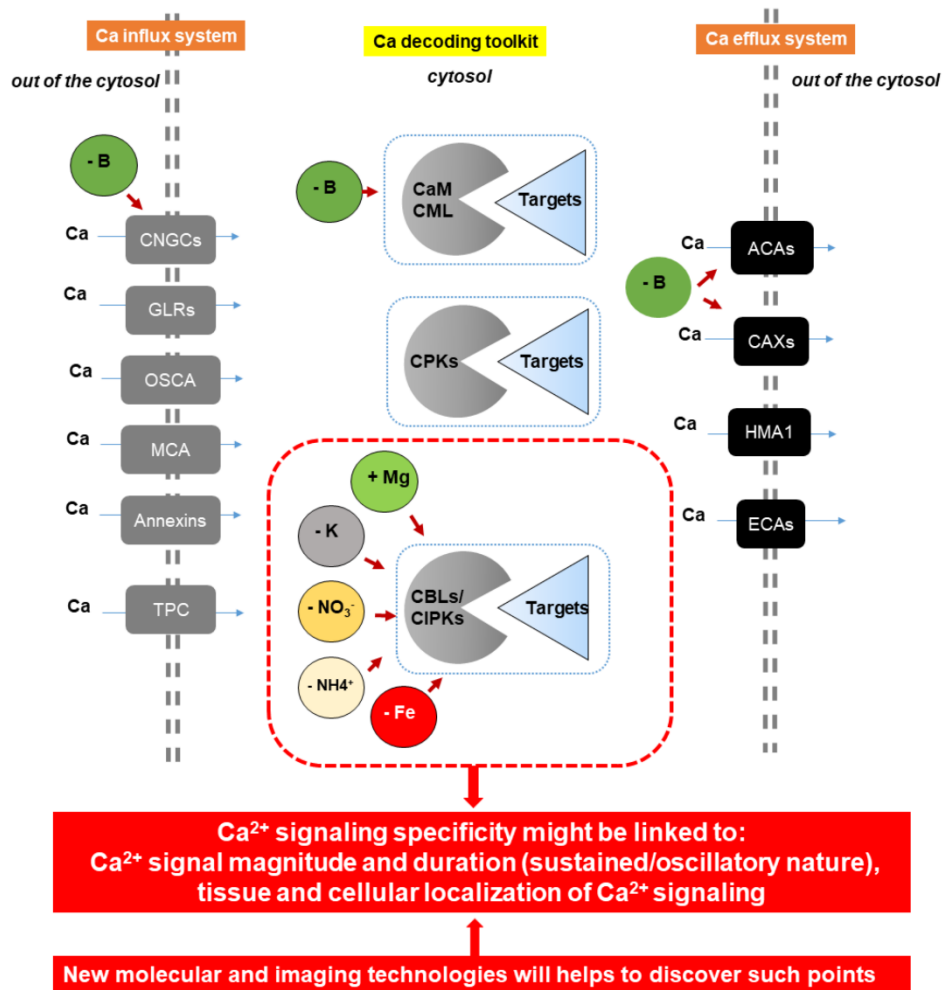
FIGURE 1 Overview of knowledge obtained about the role of Ca^{2+} sensing and signaling processes in the homeostasis of nutrients

Proteins characterizing the three steps of Ca^{2+} signaling are reported as follows: Ca^{2+} influx transporters (on the left) Ca^{2+} decoding proteins (on the centre) and Ca^{2+} efflux proteins (on the right). So far the involvement of proteins belonging to Ca^{2+} signaling has been identified under specific nutrients starvation (-) or excess (+) conditions indicated with the circles. Abbreviations: Cyclic nucleotide gated channels (CNGCs); Glutamate receptor-like channels (GLRs); Two pore channels (TPCs); Mechanosensitive channels (MCAs); Reduced hyperosmolality-induced $[Ca^{2+}]_{cyt}$ increase (OSCAs); Autoinhibited Ca^{2+}-ATPase (ACAs); ER-type Ca^{2+}-ATPase (ECAs); P1-ATPase (e.g. HMA1); Ca^{2+} exchangers (CAX); Calmodulin (CaM); CaM-like proteins (CMLs); Calcineurin B-like proteins (CBL); Ca^{2+}-dependent protein kinases (CDPKs or CPKs); CBL proteins form functional complexes with CBL interacting protein kinases (CIPKs).
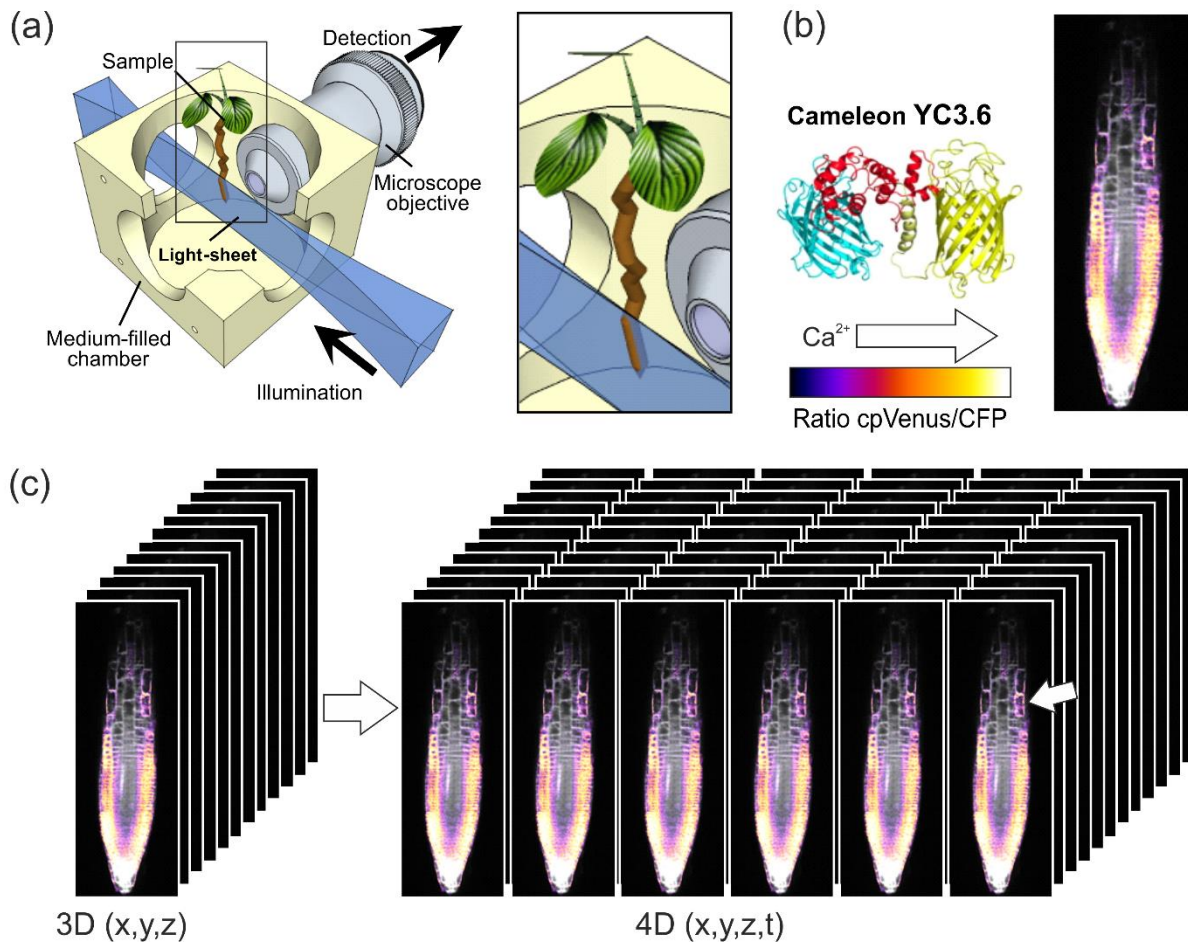
FIGURE 2 Light Sheet Fluorescence Microscopy (LSFM) allows long-term vertical imaging of plants expressing genetically encoded Ca^{2+} sensors

(a) schematic diagram of LSFM setup with the plant placed vertically. (b) Example of a root tip of an *Arabidopsis thaliana* root tip expressing the Cameleon YC3.6 sensor imaged with LSFM. The false colour (fire LookUp Table) on the root tip represents the YC3.6 cpVenus/CFP ratio which reports the levels of Ca^{2+} in the different cell types. (c) A 3D (x,y,z) acquisition if repeated at different time points generates a 4D (x,y,z,t) acquisition which allows the Cameleon ratio to be visualized at the level of single cell (small white arrow).

TABLE 1 Summary of available fluorescent-based Ca^{2+} genetically encoded sensors used in plants

Name	Version	Type	Peaks of Excitation/Emission (mn)	*In vitro* KD for Ca^{2+}*	Subcellular localisation	References
Cameleon	YC3.6	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	250 nM	cytosol and nucleus	Nagai *et al.*, 2004; Mori *et al.*, 2006
	NES-YC3.6	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	250 nM	cytosol	Krebs *et al.*, 2012
	NLS-YC3.6	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	250 nM	nucleus	Krebs *et al.*, 2012
	NUP-YC3.6	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	250 nM	nucleus	Costa *et al.*, 2017
	4mt-YC3.6	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	250 nM	mitochondria	Loro *et al.*, 2012
	PM-YC3.6-LTI6b	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	250 nM	plasma membrane	Krebs *et al.*, 2012
	2Bam4-YC3.6	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	250 nM	chloroplasts and plastids	Loro *et al.*, 2016
	Nano65	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	65 nM	cytosol and nucleus	Horikawa *et al.*, 2010; Choi *et al.*, 2014
	SP-YC4.6-ER	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	58 nM / 14.4 μM	endoplasmic reticulum	Nagai *et al.*, 2004; Iwano *et al.*, 2009
	2Bam4-YC4.6	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	58 nM / 14.4 μM	chloroplasts and plastids	Loro *et al.*, 2016
	4mt-D3cpv	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	600 nM	mitochondria	Loro *et al.*, 2013
	D3cpv-KVK-SKL	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	600 nM	peroxisomes	Palmer *et al.*, 2006; Costa *et al.*, 2010
	TP-D3cpv	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	600 nM	tonoplast	Krebs *et al.*, 2012
	CRT-D4ER	Ratiometric CFP/Citrine	Ex 440/Em 480/530	195 μM	endoplasmic reticulum	Palmer *et al.*, 2006; Bonza *et al.*, 2013
Twitch	Twitch 3	Ratiometric CFP/cpCit174	Ex 440/Em 480/530	250 nM	cytosol and nucleus	Thestrup *et al.*, 2014; Waadt *et al.*, 2017
Geco	R-Geco1	Intensiometric mApple	Ex 561/Em 600	482 nM	cytosol and nucleus	Zhao *et al.*, 2011; Ngo *et al.*, 2014; Keinath *et al.*, 2015
	NR-Geco1	Intensiometric mApple	Ex 561/Em 600	482 nM	nuclear	Zhao *et al.*, 2011; Kelner *et al.*, 2018
	NR-Geco1.2	Intensiometric mApple	Ex 561/Em 600	1.2 μM	nuclear	Wu *et al.*, 2013; Kelner *et al.*, 2018
	CG-Geco1	Intensiometric cpGFP	Ex 488/Em 515	749 nM	cytosol	Zhao *et al.*, 2011; Kelner *et al.*, 2018
	CG-Geco1.2	Intensiometric cpGFP	Ex 488/Em 515	1.15 μM	cytosol	Zhao *et al.*, 2011; Kelner *et al.*, 2018
	R-Geco1-mTurquoise	Ratiometric mApple/mTurqouise	Ex 405/561/Em 480/600	NA	cytosol and nucleus	Waadt *et al.*, 2017
GCaMP	GCaMP3	Intensiometric cpGFP	Ex 488/Em 515	542 nM	cytosol and nucleus	Tian et al., 2009; Vincent *et al.*, 2017
	GCaMP6f	Intensiometric cpGFP	Ex 488/Em 515	375 nM	cytosol and nucleus	Chen et al., 2013; Waadt *et al.*, 2017
	GCaMP6s	Intensiometric cpGFP	Ex 488/Em 515	144 nM	cytosol and nucleus	Chen et al., 2013; Liu *et al.*, 2017
	MatryoshCaMP6s	Ratiometric cpGFP/LSSmOrange	Ex 440/Em 515/600	197 nM	cytosol and nucleus	Ast *et al.*, 2017
Case	Case12	Intensiometric cpGFP	Ex 488/Em 515	1 μM	cytosol and nucleus	Souslova *et al.*, 2007; Zhu *et al.*, 2013

The in vitro KD for Ca^{2+} of the different sensors are those reported in the original works. Other available recently generated lines are reported in Waadt, Krebs, Kudla, & Schumacher (2017). Table modified from ref. Costa, Navazio, & Szabo (2018) with the permission of Oxford University Press.

TABLE 2 Microscope solutions for the *in vivo* Ca^{2+} imaging by means of genetically encoded sensors in response to nutrient stimuli

	Single Cell	Optical sectioning	Long-term imaging	Temporal resolution	Spatial resolution	4D analysis	Vertical position	Perfusion	Advantageous Cost
WF	1	0	2	3	1	0	1	3	3
LSCM	3	3	2	2	3	2	1	3	2
SD	3	3	2	3	2	3	1	3	1
LSFM	3	3	3	3	2	3	3	3	3

The table reports different microscopy approaches that can be used for the analysis of Ca^{2+} dynamics/levels in plant cells using genetically encoded biosensors. The score, from 0 to 3 (0 means worst and 3 means best), is based on our direct experience taking into considerations technical aspects previously considered in a recent publication (Ovečka et al., 2018). Wide-field (WF) can allow single cell resolution in leaf epidermal strips but not in complex intact organs like leaf or root cells. In this latter case there is the need of optical sectioning which is offered by a laser scanner confocal microscope (LSCM), a spinning disk confocal (SD) or Light Sheet Fluorescence Microscopy (LSFM). WF can allow to perform long-term imaging similarly to the two confocal techniques. However, the possibility to perform vertical imaging in plants growing inside tubes filled with a jellified medium with LSFM guarantees better performances, limiting possible stresses. The temporal resolution considered as the speed of acquisition of stacks (with LSCM, SD or LSFM) or entire root is relatively good with all systems. However, the LSCM is indeed the slower. The fact that the WF microscope does not offer optical sectioning cannot allow to perform 4D imaging (x,y,z,t) which is possible with the other systems. In this latter case the slower acquisition of LSCM might represent a limit. The LSFM offers the easiest solution to perform imaging with plants grown vertical (see text). However, all traditional microscopes could be potentially placed in a vertical position (von Wangenheim et al., 2017). With all the microscopes a perfusion system for nutritional solution exchange can be installed. The WF is relatively inexpensive for both its purchase and to run the experiments (e.g. very long-term imaging, up to 2500 hours, could be done with a single fluorescent lamp and more with LED lights). LSCM and SD are expensive systems for both purchase and the imaging facility fees for their use. LSFM can be relatively expensive if purchased from a company, but can also be built starting purchasing, lasers, optics and camera/s and assembling them following Wiki tutorials like the OpenSPIM (https://openspim.org/Welcome_to_the_OpenSPIM_Wiki) (Costa, Candeo, Fieramonti, Valentini, & Bassi, 2013; Pitrone et al., 2013).