Harnessing the new emerging imaging technologies to uncover the role of Ca2+ signalling in plant nutrient homeostasis

Abstract
Increasing crop yields by using ecofriendly practices is of high priority to tackle problems regarding food security and malnutrition worldwide. A sustainable crop production requires a limited use of fertilizer and the employment of plant varieties with improved ability to acquire nutrients from soil. To reach these goals, the scientific community aims to understand plant nutrients homeostasis by deciphering the nutrient sensing and signalling mechanisms of plants. Several lines of evidence about the involvement of Ca2+ as the signal of an impaired nutrient availability have been reported. Ca2+ signalling is a tightly regulated process that 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 Ca2+ signalling in the homeostasis of nutrients. In this review, we present new emerging technologies, based on the use of genetically encoded Ca2+ sensors and advanced microscopy, which offer the chance to perform in planta analyses of Ca2+ 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.

1 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 and 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 ecofriendly 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 et al., 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), and sulfur (S)—and into the micronutrients—chlorine (Cl), boron (B), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni), and molybdenum (Mo; White et al., 2017; White & Brown, 2010). 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 remobilization of the nutrients (Baxter, 2015; Baxter et al., 2008; Briat, Rouached, Tissot, Gaymard, & Dubos, 2015; Coubert et al., 2019; Lahner et al., 2003). Because some elements display chemical analogy or similarities among each other, the ionome composition of plants is determined by the multilevel interactions occurring among nutrients (Baxter et al., 2008). Such similarity can be observed for some elements at the transport level. For example, the IRON-REGULATED TRANSPORTER 1 (IRT1) can take up Fe2+ as well as other divalent cations such as Mn2+, Co2+, Zn2+, and cadmium (Cd2+; Baxter et al., 2008).

Generally, as occurs under different environmental conditions, regulation of plant response to nutrient starvation occurs at different levels: (a) perception of external/internal nutrient status (nutrient sensing); (b) amplification of the perceived stimulus through transduction mechanisms (nutrient signalling); (c) activation of acclimation responses to the stress. Whereas acclimation responses to nutrient starvation represent the most investigated issue in the plant mineral nutrition field, the nutrient sensing and signalling 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 (NO3−) 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/signalling pathways of perceived nutrient imbalance in plants represents a challenging issue. As recently highlighted in Wilkins, Matthus, Swarbreck, and Davies (2016), Ca2+-mediated signalling appears to play a central role in the regulation of nutrient deficiency stress. Here, the recent advances in this scientific area and the role of the new emerging imaging technologies for studying will be discussed.

2 THE ROLE OF Ca2+ AS SECOND MESSENGER

Calcium ions (Ca2+) are ubiquitous intracellular signalling messengers (Clapham, 2007). The origin of the signalling functions of Ca2+ 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 Ca2+ concentrations at low nM ranges (100–200 nM), which becomes crucial for Ca2+-based signalling (Clapham, 2007). In plants, rapid and transient increase of cytosolic Ca2+ concentration mediated by the opening of Ca2+-permeable channels (e.g., GLRs, CNGCs, OSCAs, MCAs, Annexins, and potentially TPC1) or by downregulation of Ca2+-export systems (e.g., ACAs and ECAs Ca2+-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 Ca2+ concentration that function as a versatile second messenger (Clapham, 2007; Kudla et al., 2018; Sanders, Pelloux, Brownlee, & Harper, 2002). In plant cells, different stimuli are associated with specific intracellular Ca2+ concentration changes characterized by given amplitudes and durations that might be decoded by different cellular Ca2+ sensors (see, e.g., Evans, McAinsh, & Hetherington, 2001; Monshausen, 2012; Sanders et al., 2002; Trewavas, Read, Campbell, & Knight, 1996; Whalley & Knight, 2013; Xiong et al., 2006). Plants possess many proteins predicted to bind Ca2+ through EF domains (nearly 250

in Arabidopsis; Day, Reddy, Shad Ali, & Reddy, 2002; DeFalco, Bender, & Snedden, 2009). Calmodulin (CaM; five 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 Ca2+ binding proteins that work as Ca2+ sensors/responders (reviewed in DeFalco et al., 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 Ca2+ in a signalling process on the basis of the evidence that an applied stimulus, or a given growth condition, affects the resting cytosolic Ca2+ concentrations. In addition, forward and reverse genetic screenings that led to the identification of genes coding for Ca2+ binding proteins put Ca2+ into the signalling game.

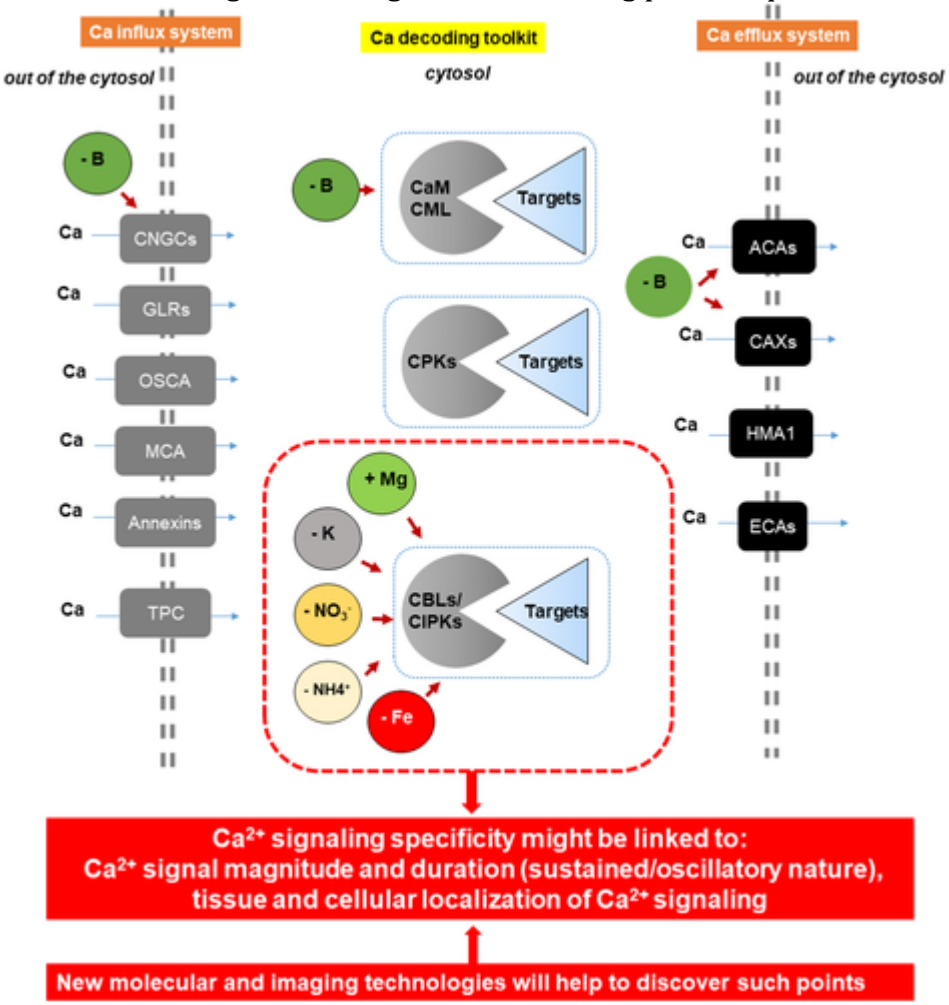

Figure 1

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Overview of knowledge obtained about the role of Ca2+ sensing and signalling processes in the homeostasis of nutrients. Proteins characterizing the three steps of Ca2+ signalling are reported as follows: Ca2+ influx transporters (on the left) Ca2+ decoding proteins (on the centre) and Ca2+ efflux proteins (on the right). So far, the involvement of proteins belonging to Ca2+ signalling 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 [Ca2+]cyt increase (OSCAs); autoinhibited Ca2+-ATPase (ACAs); ER-type Ca2+-ATPase (ECAs); P1-ATPase (e.g., HMA1); Ca2+ exchangers (CAX); calmodulin (CaM); CaM-like proteins (CMLs); calcineurin B-like proteins (CBL); Ca2+-dependent protein kinases (CDPKs or CPKs); CBL proteins form functional complexes with CBL interacting protein kinases (CIPKs) [Colour figure can be viewed at wileyonlinelibrary.com]

3 METHODS TO MEASURE Ca2+ DYNAMICS IN VIVO

To hypothesize whether Ca2+ plays a role in plant nutritional signalling, the study of its in vivo intracellular concentration/homeostasis should be addressed. This is now possible thanks to molecular and imaging technologies. Analysis of Ca2+ dynamics in living plants was initially addressed by using Ca2+ 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 Ca2+-signalling 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 noninvasive monitoring of free Ca2+ levels, enabling real-time, spatially and temporally resolved imaging of Ca2+ levels in different cell types and subcellular compartments (reviewed in Costa & Kudla, 2015; Costa et al., 2018; Stael et al., 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 Ca2+ 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 Ca2+ 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 Ca2+ 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 Ca2+ signalling 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 Ca2+ 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 (nonratiometric 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), which 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 Ca2+-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 rely on a change in the sensor quantum yield, measured as a change of the intensity of the emitted fluorescence that depends primarily by the amount of Ca2+ 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 Ca2+ 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). For the sake of clarity, we can state that intensiometric biosensors exhibit a higher signal change compared with a FRET-based sensor (e.g., Cameleon) in response to several stimuli (Keinath et al., 2015; Kleist et al., 2017; Nguyen, Kurenda, Stolz, Chételat, & Farmer, 2018; Toyota et al., 2018; Vincent et al., 2017; Waadt et al., 2017). However, at the present time, the Cameleon sensor, thanks to its FRET-based ratiometric properties, has shown to be particularly suited for the quantification of Ca2+ levels in different genetic backgrounds (wild type vs. mutants; Behera et al.,2018; Corso, Doccula, de Melo, Costa, & Verbruggen, 2018; Costa et al., 2017; 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 Ca2+ dynamics/homeostasis in different nutritional conditions. Remarkably, Cameleon and some intensiometric sensors are also available for the analyses of Ca2+ dynamics in different intracellular compartments such as mitochondria, chloroplasts/plastids, endoplasmic reticulum, nucleus, and peroxisomes (Table 1; reviewed in Costa et al., 2018).

Table 1. Summary of available fluorescent-based Ca2+ genetically encoded sensors used in plants

Name	Version	Type	Peaks of excitation/ emission (mn)	In vitro KD for Ca2+** The in vitro KD for Ca2+ of the different sensors are those reported in the original works. Other available recently generated lines are reported in Waadt et al. (2017). Table modified from Costa et al. (2018) with the permi	Subcellular localization	References

				ssion of Oxford University Press.		
Cameleon	YC3.6	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	250 nM	Cytosol and nucleus	Mori et al., 2006; Nagai et al., 2004
	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	Choi, Toyota, Kim, Hilleary, & Gilroy, 2014; Horikawa et al., 2010
SP-YC4.6-ER	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	58 nM / 14.4 µM	Endoplasmic reticulum	Iwano et al., 2009; Nagai et al., 2004
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, Ruberti, Zottini, & Costa, 2013
D3cpv-KVK-SKL	Ratiometric CFP/cpVenus	Ex 440/Em 480/530	600 nM	Peroxisomes	Costa et al., 2010; Palmer et al., 2006
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	Bonza et al., 2013; Palmer et al., 2006
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	Keinath et al., 2015; Ngo et al., 2014; Zhao et al., 2011
	NR-Geco1	Intensiometric mApple	Ex 561/Em 600	482 nM	Nuclear	Kelner et al., 2018; Zhao et al., 2011
	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	Kelner et al., 2018; Zhao et al., 2011
	CG-Geco1.2	Intensiometric cpGFP	Ex 488/Em 515	1.15 µM	Cytosol	Kelner et al., 2018; Zhao et al., 2011
	R-Geco1-mTurquoise	Ratiometric mApple/mTurqouise	Ex 405/561/Em 480/600	NA	Cytosol and nucleus	Waadt et al., 2017
	GCaMP3	Intensiometric cpGFP	Ex 488/Em 515	542 nM	Cytosol and nucleus	Tian et al., 2009; Vincent et al., 2017
GCaMP	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

	Matryoshka CaMP6s	Ratiometric cpGFP/LSS mOrange	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., 2014

Note.
* The in vitro KD for Ca2+ of the different sensors are those reported in the original works. Other available recently generated lines are reported in Waadt et al. (2017). Table modified from Costa et al. (2018) with the permission of Oxford University Press.
In conclusion, nowadays, we can choose among plant lines expressing different types of Ca2+ sensors, the choice of which can be made based on the specific experiment we want to carry out (see Table 1 and Costa et al., 2018).

4 INTRODUCTION OF Ca2+ SIGNALLING IN MACRONUTRIENT NUTRITION

Recent results have identified Ca2+ signalling components in the sensing of nutrients availability. Here, we summarize the recent findings related to K, N, P, and Mg homeostasis.

4.1 K homeostasis

One of the first examples showing an involvement of Ca2+ 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 (Batistic, 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+ (Batistic et al., 2010; Xu et al., 2006). 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 Ca2+-based signalling. However, the authors of both works did not show the occurrence of any low K+-induced cytosolic Ca2+ increase. However, 10 years later, the group of Joerg Kudla, thanks to high resolution in vivo Ca2+ imaging based on the use of Arabidopsis seedlings expressing the Cameleon YC3.6 (see above; Krebs et al., 2012; Nagai et al., 2004) was able to provide evidence that a quick drop in K+ availability (from 5 to 0 mM) triggered a transient cytosolic Ca2+ 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 Ca2+ 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., Cuéllar 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 Ca2+ 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 KINASE 1 that in turn is an interactor of the Ca2+ sensor CML9, thus suggesting another possible level of HAK5 regulation mediated by Ca2+-based regulation (Brauer et al., 2016). Thus, Ca2+-based signalling can regulate the

activity of K+ transporters in several different ways. We might speculate that different Ca2+ signatures could activate different Ca2+ sensors and regulate differentially the same or different target/s (Kudla et al., 2018; Sanders et al., 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 Ca2+ dynamics in response to different K+ availability.

AKT1 and HAK5 are not the only K+ transport mechanisms involved in the K+ nutrition regulated by Ca2+-dependent processes. Other channels like AKT2 or transporters like Two Pore K (TPK) are posttranscriptionally regulated by Ca2+. AKT2 is a potassium channel playing a key role in plant nutrition because 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 Ca2+ 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 Ca2+ 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 Ca2+. Thus, cytosolic Ca2+ transients evoked by K+ depletion can potentially also affect TPKs, of which the activity is linked to nutrient remobilization and K+ homeostasis (Gobert et al., 2007).

4.2 N homeostasis

Another important macronutrient for plants is nitrogen (N). Nitrogen is adsorbed from the soil in two forms, as nitrate (NO3−) or ammonium (NH4+) ions with a preference to NO3−, because NH4+ 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 Ca2+-mediated signalling, because in Arabidopsis, the CBL1/9-CIPK23 complex can phosphorylate both the CHL1/NRT1.1/NPF6.3 NO3− transceptor and the AMT1;1 NH4+ transporter (Ho et al., 2009; Straub, Ludewig, & Neuhäuser, 2017). Similarly to what was discovered for the AKT1-mediated K+ uptake, the NO3− 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 et al., 2009).

4.3 N homeostasis: NO3−

Using Arabidopsis plants expressing aequorin, it was demonstrated that plants grown in the presence of NH4+ as the sole N source and then exposed to mM concentrations of NO3− showed an immediate and transient cytosolic Ca2+ increase, which requires the activity of the phospholipase C enzyme (Riveras et al., 2015). Such NO3−-induced cytosolic Ca2+ increase may be ultimately responsible for the activation of the CBL-CIPK Ca2+ sensor and therefore the CHL1/NRT1.1/NPF6.3 regulation (Riveras et al., 2015). Following a different approach,

the laboratory of Jen Sheen using the ultrasensitive Ca2+ sensor CGaMP6s (Chen et al., 2013) confirmed the existence of a NO3−-induced Ca2+ 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 NO3− 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 signalling, hormones, and proliferation (Liu et al., 2017). Overall a model can be predicted in which NO3− induces a cytosolic Ca2+ transient that regulates its own transport through a CBL1/9-CIPK23 mediated mechanism and the gene transcription through CPK10, CPK30, and CPK32 (Ho et al., 2009; Liu et al., 2017). To make the scenario even more complex, there is also the demonstration that among the NO3−-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) POLYMERASE 1 (FIP1) transcription factor (Wang et al., 2018). Moreover, CIPK8 also regulates posttranscriptionally the activity of CHL1/NRT1.1/NPF6.3 NO3− transporter (Ho et al., 2009).
The CHL1/NRT1.1/NPF6.3, beside its direct role in transporting NO3− 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 signalling (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 Ca2+ increase and thus potential Ca2+ sensors activation, but it has been shown to affect Ca2+-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 Ca2+ responses to extracellular ATP, sorbitol, and NaCl compared with control (Matthus et al., 2019). Remarkably, this change in stimulus-induced cytosolic Ca2+ 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 Ca2+ as a signal transducer.

4.4 N homeostasis: NH4+

The NH4+ form is transported into the cells by high-affinity NH4+ transporters (AMTs; Xu et al., 2012), and to avoid toxic accumulation of cytoplasmic NH4+, the AMT transporter activity must be finely regulated. In Arabidopsis thaliana, AMTs (AMT1;1 and AMT1;2) are efficiently inactivated by phosphorylation (Straub et al., 2017), and surprisingly, the kinase responsible for such inactivation is the CIPK23 in complex with CBL1 (Straub et al., 2017). Interestingly, NH4Cl treatment was shown to induce cytosolic Ca2+ 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 NH4+-induced cytosolic Ca2+ increase, but we might tentatively speculate that because external NH4+ can inhibit the high-affinity K+ transport (Hirsch, Lewis, Spalding, & Sussman, 1998), the NH4+-induced cytosolic Ca2+ increase might be mediated by K+-deprivation (Behera et al., 2017).

In summary, the transport of K+, NO3−, and NH4+ is highly regulated and interconnected, and this connection is possibly dependent on Ca2+ signalling, mainly through the activity of CBL1/9-CIPK23 complexes.

4.5 Mg homeostasis

A further macronutrient that is linked to Ca2+ homeostasis and signalling is magnesium (Mg2+). Mg2+ is an essential macronutrient for plants, because 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 Mg2+ present in the soil are deleterious to plant growth, thus affecting crop production. Members of the Mg2+ transporters (MGTs) family have been identified to facilitate Mg2+ transport in Arabidopsis (Li, Tutone, Drummond, Gardner, & Luan, 2001). In yeast, Mg2+ deprivation elicits rapid Ca2+ influx and activates a Ca2+/calcineurin signalling 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 Mg2+ (Visscher et al., 2010), some studies established a critical function of Ca2+ signalling in plant adaptation to high Mg2+ stress (Tang et al., 2015). Two tonoplast-CBL proteins, CBL2 and CBL3, are predicted to perceive a transient rise of Ca2+ in the cytosol under Mg2+ excess conditions. After a putative Ca2+ signal, CBL2 and CBL3 activate a subset of CIPKs, such as CIPK3, 9, 23, and 26, which in turn phosphorylate the Mg2+ transport systems of the tonoplast, allowing vacuolar Mg2+ sequestration and avoiding the accumulation of toxic levels of Mg2+ in the cytosol (Tang et al., 2015). So far, the Mg2+ transporters localized at the tonoplast involved in such a mechanism are still unknown, as the AtMXT, a Mg2+/H+ antiporter (Amalou, Gibrat, Brugidou, Trouslot, & d'Auzac, 1992) as well as the MGT2 and MGT3 transporters, tonoplast-localized Mg2+ transporters (Conn et al., 2011) have been not involved in the Ca2+-mediated vacuolar sequestration of Mg2+ under excess conditions (Tang et al., 2015).

5 Ca2+ SIGNALLING IN MICRONUTRIENT HOMEOSTASIS

Ca2+-mediated signalling has also been involved in the regulation of plant responses to micronutrient starvation. Among micronutrients, information collected concerns mainly Fe and B.

5.1 Fe homeostasis

In dicot plants the Fe uptake mechanism is based on a reduction-based strategy, where the ferric form (Fe3+) of Fe is reduced at the root surface and imported as ferrous Fe (Fe2+; 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 IRT1 (Colangelo & Guerinot, 2004). Tian et al. (2016) found that Arabidopsis seedlings grown for 4 days in Fe deficiency conditions showed an increase of cytosolic Ca2+ 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 Ca2+ increase in the early root differentiation zone, both in the central cylinder and in the epidermis. Remarkably, Tian et al. (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 Ca2+ 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 Ca2+ (Khan et al., 2019). Further evidence of a link between Ca2+ signalling 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 homodimers 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 Ca2+ 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 Ca2+ influx across the plasma membrane, which, in turn, may lead to a cytosolic Ca2+ increase through the activation of ROS-activated ion channels (Demidchik, 2018; Laohavisit et al., 2012; 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 Ca2+ signature after exogenous application of ATP in Pi-starved root tips (Matthus et al., 2019). All these findings indicated that (a) Ca2+ signalling is directly involved in the transduction of Fe deficiency signals and thereby in the activation of stress responses and that (b) impaired Fe content might affect the ROS/Ca2+ hub in signalling processes under Pi starvation.

5.2 B homeostasis

Upon B deprivation, an increase of Ca2+ 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 Ca2+-signalling proteins involved in Ca2+ transport, sensing, and decoding was upregulated (Quiles-Pando et al., 2013). The involvement of Ca2+ permeable channels, responsible for the rise of Ca2+ concentration in the cytosol under B starvation, was hypothesized by the fact that lanthanum, a nonselective blocker of cation channels and stretch-activated Ca2+-permeable channels (reviewed in De Vriese, Costa, Beeckman, & Vanneste, 2018), inhibited the cytosolic Ca2+ increase as well as an upregulation of the genes coding for the CNGC19 (a putative Ca2+-permeable channel). Furthermore, the sensitivity of Ca2+ transport to diphenyleneiodonium (DPI) suggested an involvement of NADPH oxidase in the modulation of responses under B deficiency (Koshiba et al., 2010). In the same work, the authors also demonstrated that B deficiency induced the expression of genes coding for three auto-inhibited Ca2+-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 Ca2+

into the apoplast and vacuole, respectively, to possibly reestablish the cytosolic Ca2+ resting after a transient increase. Interestingly, the increased Ca2+ 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 favour a Ca2+ 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 Ca2+-dependent protein kinases (CPK28, CPK29), involved in Ca2+ sensing and decoding process, respectively, were also upregulated 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 as those belonging to WRKY and MYB TFs families (González-Fontes et al., 2016; Wilkins et al., 2016). Indeed, an upregulation of some WRKY, MYB TFs was observed under B deficiency (González-Fontes et al., 2016)

6 NEW MOLECULAR AND IMAGING TECHNOLOGIES TO TEST NEW HYPOTHESES IN Ca2+-BASED PLANT NUTRITION SIGNALLING

As mentioned above, the study of in vivo Ca2+ dynamics/homeostasis in living plants, cultivated in different growth conditions, was made possible thanks to modern microscopy technologies. Whereas in its infancy in vivo Ca2+ 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 et al., 2017; Behera & Kudla, 2013; Candeo, Doccula, Valentini, Bassi, & Costa, 2017; Costa et al., 2017; Costa, Candeo, Fieramonti, Valentini, & Bassi, 2013; Keinath et al., 2015; Kelner et al., 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 et al., 2017). The possibility of pursuing Ca2+ imaging analyses in root cells represents an important tool to understand the role of Ca2+ signalling 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 that can help with the identification of the specific nutrient sensor (Table 2). For example, K+ and Fe deprivation triggered a cytosolic Ca2+ increase in the postmeristematic stelar tissue of the Arabidopsis root elongation zone (Behera et al., 2017; Gratz et al., 2019; Tian et al., 2016). B starvation and NO3− administration affected the Ca2+ levels in the root tip meristematic cells (Liu et al., 2017; Quiles-Pando et al., 2013). Pi deprivation altered the Ca2+ 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 Ca2+ 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 Ca2+-signalling 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, and cortex) 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 Ca2+ dynamics in the phloem of intact Arabidopsis leaves (Toyota et al., 2018; Vincent et al., 2017). Besides the study of the tissue specificity of nutrient-induced cytosolic Ca2+ responses, another important consideration is the design of a more physiological experimental approach. In fact, most of the Ca2+ 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 et al., 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 Ca2+ 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 Ca2+. Thus, it would be significant to carry out experiments in which the monitoring of Ca2+ 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 et al., 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 et al. (2011) was the first group that 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 et al., 2011). A few years later, we implemented the LSFM technique for the analysis of stimulus-induced Ca2+ dynamics (Costa et al., 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 Ca2+ dynamic analyses were possible in untouched, vertically grown roots (Costa et al., 2013). Later on, we carried out analyses of root hair tip Ca2+ oscillations in plants grown in standard growth medium or supplemented with 50 nM of auxin (NAA; Candeo et al., 2017). Based on these new possibilities, it will be

therefore desirable to design new experiments in which plants expressing a Ca2+ 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 Ca2+, but also the analysing of the morphology and quantifying the size of the cells. Additionally, LSFM would help to explore how nutrient deprivation affects Ca2+ levels in subcellular compartments. In fact, the availability of transgenic lines expressing Ca2+ biosensors in mitochondria, plastids, endoplasmic reticulum, and so on (reviewed in Costa et al., 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 with an 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, Ca2+ 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 Ca2+ 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 (Candeo et al., 2017; Maizel et al., 2011) and the cells must be illuminated with a laser, a condition that is not found in nature and that can potentially have some effects on cell physiology.

Table 2. Microscope solutions for the in vivo Ca2+ 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

Note. The table reports different microscopy approaches that can be used for the analysis of Ca2+ 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 et al., 2013; Pitrone et al., 2013).

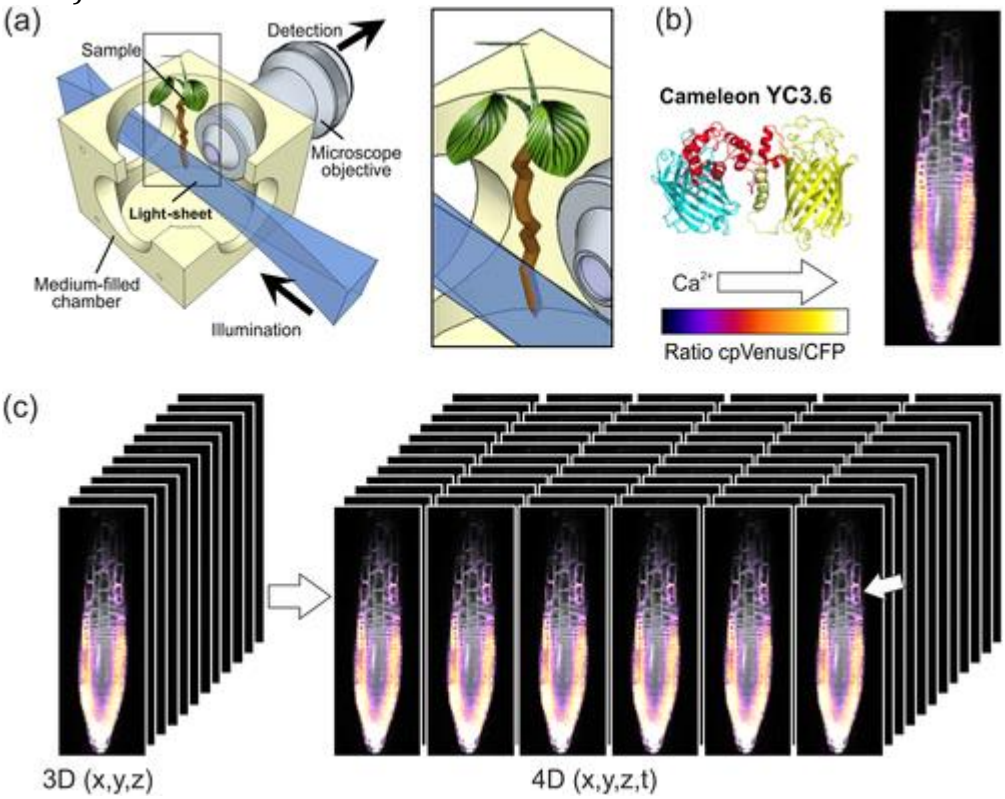

Figure 2
Open in figure viewerPowerPoint
Light sheet fluorescence microscopy (LSFM) allows long-term vertical imaging of plants expressing genetically encoded Ca2+ sensors. (a) Schematic diagram of LSFM set-up with the

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 Ca2+ 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 that allows the Cameleon ratio to be visualized at the level of single cell (small white arrow) [Colour figure can be viewed at wileyonlinelibrary.com]

7 FUTURE PERSPECTIVES IN THE SIGNALLING 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 Ca2+ signalling 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 Ca2+-based signalling process in response to nutritional stress. The basic Ca2+-signalling components can be considered as follows: (a) Ca2+ influx system (responsible for the increase of Ca2+ concentration in the cytosol after stimulus sensing), (b) Ca2+-decoding toolkit (responsible for the Ca2+-mediated signal transduction), and (c) Ca2+ efflux systems (responsible for the restoration of Ca2+ 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 Ca2+ decoding process, whereas only very little evidence is available about the specific Ca2+ influx and efflux systems involved in the generation and regulation of cytosolic Ca2+ increases (Figure 1). To fill this gap, the study of Ca2+ signalling in response to nutritional stress, in different genetic backgrounds of known mutants for Ca2+ export systems (e.g., Ca2+-ATPase or CAXs) or putative Ca2+ permeable channels, might provide useful clues. Besides, forward genetic screenings might be pursued through the mutagenesis of plant lines expressing Ca2+ sensors, scoring for plants showing altered Ca2+ homeostasis in given nutritional conditions. Thus, pursuing a Ca2+-sensor-based screening in nutrient stress conditions might allow us to identify new signalling components possibly related to Ca2+ transport.

An interesting observation that raises fundamental questions is that except for B, altered homeostasis of different nutrients require the activity of CBL-CIPKs complexes (especially CIPK23) as the Ca2+ 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 et al., 2015, and references therein). Therefore, we might suggest that different nutrient stresses can engage similar components of the Ca2+ signalling process, depending on the existing cross-talk occurring among the nutrients themselves. However, such observations raise the question of how the transduction signalling might be specific for a given nutrient. Overall, the Ca2+ signalling 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 Ca2+, might be related to several features of the Ca2+ signal itself, like its magnitude, duration, localization, and sustained/oscillatory nature, which can potentially activate different Ca2+ sensors (Burgoyne & Haynes, 2015; Kudla et al., 2010; Sanders et al., 2002). 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 Ca2+ 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 crosstalk and Ca2+ signalling by increasing the analytical sensitivity to monitor the above-mentioned features of Ca2+ 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 Ca2+-mediated responses, as well as a precise temporal progression of signal transduction within a plant. Moreover, we might also consider pursuing analysis of Ca2+ dynamics at the subcellular level (e.g., by using plant lines expressing Ca2+ 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, because (a) they require several nutrients to keep metabolic processes working and (b) 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 Ca2+ in the mitochondrial matrix (Teardo et al., 2017) or in plastidial stroma (Frank et al., 2019); thus, mutants for these transporters might be studied to understand whether they are involved in the regulation of cellular Ca2+ homeostasis. The fact that several indications show that organelles also play a role in the regulation of nuclear genes expression under nutrient starvation (Vigani et al., 2016; Vigani, Solti, Thomine, & Philippar, 2019; Vigani, Zocchi, Bashir, Philippar, & Briat, 2013) 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 Ca2+ homeostasis. The combination of the use of new imaging technologies with Ca2+ 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.

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CONFLICT OF INTEREST
The authors have declared that no competing interests exist.
AUTHOR CONTRIBUTIONS
G. V. and A. C. carried out the literature study, made the figures, and wrote the manuscri

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