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Short communication

Zn deficiency in *Brassica napus* induces Mo and Mn accumulation associated with chloroplast proteins variation without Zn remobilization

Vincent Billard [a, b, c], Anne Maillard [a, b, c], Maria Garnica [d], Florence Cruz [e], José-Maria Garcia-Mina [d], Jean-Claude Yvin [e], Alain Ourry [a, b, c], Philippe Etienne [a, b, c, *]

[a] Normandie University, Caen, France
[b] UNICAEN, UMR 950 Ecophysiologie Végétale, Agronomie et nutritions N, C, S, F-14032 Caen, France
[c] INRA, UMR 950 Ecophysiologie Végétale, Agronomie et nutritions N, C, S, F-14032 Caen, France
[d] Timac Agro Spain, Poligono de Arazuri-Orcoyen Calle C n°32, 31160 Orcoyen, Spain
[e] Centre de Recherche International en Agroscience, CRIAS-TAI, Groupe Roullier, 55 boulevard Jules Verger, 35800 Dinard, France

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ABSTRACT

The importance of zinc (Zn) has been of little concern in human nutrition despite a strong decrease of this element in crops since the rise of high yielding varieties. For better food quality, Zn biofortification can be used, but will be optimal only if mechanisms governing Zn management are better known. Using Zn deficiency, we are able to demonstrate that Zn is not remobilized in *Brassica napus* (*B. napus*). Thus, remobilization processes should not be targeted by biofortification strategies. This study also complemented previous work by investigating leaf responses to Zn deficiency, especially from proteomic and ionomic points of view, showing for example, an increase in Manganese (Mn) content and of the Mn-dependent protein, Oxygen Evolving Enhancer.

1. Introduction

Although macronutrients like carbon (C), nitrogen (N) and sulphur (S) have been a focal point in agricultural management, micronutrients have been paid far less attention. However, according to the World Health Organization (Allen et al., 2006), two billion people around the world suffer from micronutrient deficiencies, causing 7.3% of disease burden.

Among the micronutrient deficiencies in humans, severe zinc (Zn) deficiency which causes symptoms such as immune defects and growth retardation (Hambidge, 2000). However, most of the symptoms that are induced by Zn deficiency can be attributed to other environmental factors present during diagnosis, and therefore a role for Zn deficiency is likely to have remained hidden. Indeed, Zn deficiency was long considered as non existent in humans with the first hypothesis on Zn deficiency arising only in 1961 (Prasad et al., 1961). Importantly, Zn is involved in lymphocyte development and differentiation (Shankar and Prasad, 1998). As a consequence, a mild Zn deficiency can have an impact on concomitant infections such as diarrhoea (Sazawal et al., 1995), pneumonia (Bhutta et al., 1999), malaria (Caulfield et al., 2004) or AIDS (Siberry et al., 2002). Thus, masked by the infection, Zn deficiency is ignored. Moreover, no biomarkers to monitor Zn deficiency have been identified that are simpler to handle than measuring Zn levels in the blood. In response, Zn supplementation in food has been suggested for avoiding deficiencies.

However, over the past 60 years, harvest quality (*i.e.* the micronutrient contents such as Fe, Cu, Mg and Zn content) has been

Abbreviations: DW, dry weight; IRMS, Isotope-Ratio Mass Spectrometry; ICP-OES, Inductively Coupled Plasma Optical Emission Spectrometry; NCED4, Nine Cis Epoxycaroyenoid Dehydrogenase 4; PDI, Protein Disulfide Isomerase; PDH, Pyruvate Dehydrogenase; IDH, Isocitrate DH; HSC70, Heat Shock Complex 70 kDa; TPI, Triose Phosphate Isomerase; VAR3, Variegate 3; GCSH, Glycine Cleavage System H; GCC, Glycine Cleavage Complex; DHAP, Dihydroxy Acetone Phosphate; GAP, Glyceraldehyde Phosphate; F1-6BP, Fructose-1,6-Bis-Phosphate; OEE, Oxygen Evolving Enhancer; MoCo, Molybdenum Cofactor; SOD, SuperOxyde Dismutase; 2-DE, 2 Dimensional Electrophoresis; Q-PCR, Quantitative Polymerase Chain Reaction.

* Corresponding author. UNICAEN, UMR 950 Ecophysiologie Végétale, Agronomie et nutritions N, C, S, F-14032 Caen, France.
E-mail addresses: vincent.billard@unicaen.fr (V. Billard), anne.maillard@unicaen.fr (A. Maillard), mgarnica@timacagro.es (M. Garnica), fcruz@roullier.com (F. Cruz), jgmina@timacagro.es (J.-M. Garcia-Mina), jcyvin@roullier.com (J.-C. Yvin), alain.ourry@unicaen.fr (A. Ourry), philippe.etienne@unicaen.fr (P. Etienne).

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reduced despite sufficient micronutrient amounts in the soil, and this is because of varietal selection that aimes to achieve higher yields (Garvin et al., 2006; Fan et al., 2008). In addition, many poor soils may lack Zn resulting in dramatic effects on agriculture. Thus, Zn deficiency affects yield and quality of harvest with either direct or indirect negative impacts on human nutrition (Andersen, 2007; Alloway, 2013). Biofortification can be an option to increase Zn in food, but this requires more knowledge, especially concerning Zn management in plants.

Due to its capacity to form tetrahedral complexes with N-, O- and particularly S-ligands, Zn plays a functional and structural role in a large number of enzyme reactions in plants and in other organisms (Vallee and Auld, 1990). Indeed, bioinformatical studies have revealed that 9% of eukaryotic proteins are likely Zn-dependent (Andreini et al., 2009). Numerous transcription factors contain a Zinc finger domain that needs Zn ions to bind a specific DNA sequence in order to initiate and promote transcription (Klug, 2010). For example, the C_2H_2 family (characterized by a specific sequence motif containing 2 cysteins and 2 histidines) contains the majority of the Zn finger proteins with 176 different proteins. Zn–Cu superoxide dismutase (Zn–Cu SOD) also needs a Zn atom in its functional core to detoxify free radicals in cells (Abreu and Cabelli, 2010). Moreover, a lot of other key proteins are known to be regulated by Zn, such as Fructose 1,6 bisphosphatase (Shrotri et al., 1983).

Previous high scale studies on Zn deficiency have mainly used transcriptomic approaches, showing a high impact of Zn deficiency on the regulation of transcription factors. Even though Zn finger proteins are numerous they represent only 0.7% of the total amount of proteins.

Brassica napus is an important agricultural crop that needs high levels of macronutrients (especially nitrogen (N) and sulphur (S)) to reach high yields. Thus, *B. napus* is highly sensitive to S and N deficiency with negative consequences for yield and seed quality (Dubousset et al., 2010). As a consequence, high doses of N and S fertilizers are usually used to avoid these losses of yield and the quality of harvest products. This strong need for fertilizers is partly due to the low (macro)-nutrient use efficiency (defined by the ratio of seed to plant nutrient contents) of oilseed rape. For example, the low N use efficiency is due to an inefficient endogenous N mobilization (Etienne et al., 2007; Desclos et al., 2008; Avice and Etienne, 2014) associated with leaf senescence during the vegetative stage. According to these studies, *B. napus* constitutes a relevant model with a defective agro-environmental balance that might be improved. Moreover, its genetic proximity to *Arabidospis thaliana* provides easy access to molecular tools while maintaining an agronomic relevance.

Therefore, the aim of this study is to better understand the effect of Zn deficiency on *B. napus* growth and remobilization of endogenous Zn. Moreover, an ionomic approach has been used to monitor the effect of Zn deficiency on the uptake of a selection of macro- and micro-nutrients. Finally, a proteomic approach has been performed to identify the main metabolic pathways affected by Zn deficiency.

2. Material and methods

2.1. Growth conditions

Seeds of *B. napus* var. Boheme were surface-sterilized by exposure to 80% ethanol for 30 s followed by 20% sodium hypochlorite for 10 min. Then, they were germinated on perlite over demineralized water for 2 days in the dark and 1 week under natural light in a greenhouse. Just after first leaf emergence, seedlings were transferred to a 20 L tank containing the following nutrient solution: 1.25 mM KNO_3, 1.25 mM $Ca(NO_3)_2$, 0.25 mM KH_2PO_4 0.2 mM EDTA, 2NaFe, 0.5 mM $MgSO_4$, 0.01 mM H_3BO_3, 5 μM $MnSO_4$, 0.7 μM $(NH_4)_6Mo_7O_{24}$, 0.1 μM $CoCl_2$, 0.04 μM $NiCl_2$, 0.1 mM SiO_2, 1.25 mM $CaCl_2$, 0.25 mM KCl, and 0.7 μM $CuSO_4$. Control nutrient solution also contained 0.1 mM NaOH and 3 μM $ZnSO_4$ while 3 μM Na_2SO_4 was added to the Zn-deficient nutrient solution. These nutrient solutions were renewed every two days. Plants were grown under greenhouse conditions with a thermoperiod of 20 °C/17 °C day/night and a photoperiod of 16 h. Natural light was supplemented with high pressure sodium lamps (Philips, MASTER GreenPower T400W) supplying an average photosynthetically active radiation of 280 μmol photons m^{-2} s^{-1} at canopy height. After one week of growth, plants were separated into 2 sets: control plants receiving normal nutrient solution and Zn-deficient plants receiving the Zn-deficient nutrient solution (for details, see above) over 25 days. Four independent samples each consisting of three plants were harvested at the beginning of Zn-depletion (t0) and after 25 days with (control) or without Zn (−Zn). Leaves and petioles present at the beginning of Zn-depletion (referred as “old leaves” and “old petioles”, respectively) were distinguished from leaves appearing during Zn-depletion (referred as “young leaves” and “young petioles”, respectively). At each date of harvest ($t = 0$ and $t = 25$ days), whole roots from control and Zn-deficient plants were collected. An aliquot of each tissue was weighed and dried in an oven (60 °C) for dry weight (DW) determination and ground to fine powder for IRMS and ICP-OES analysis. Remaining fresh tissues were frozen in liquid nitrogen and stored at −80 °C for proteomic analyses.

2.2. Analysis of nutrients in plant tissues

An aliquot of around 4 mg DW of each plant organ sample was placed in tin capsules for total N and S analysis using an IRMS spectrometer (Isoprime, GV Instrument, Manchester, UK) linked to a C/N/S analyser (EA3000, Euro Vector, Milan, Italy). The total amount of N or S (N_{tot} or S_{tot}) in a tissue “*i*” at a given time “*t*” is calculated as:

$$N_{tot}(\text{or } S_{tot}) = \%\, N_{i,t}(\text{or } S_{i,t}) \times DW_{i,t}/100$$

Other nutrients (K, Ca, S, P, Mg, Fe, Na, Mn, B, Si, Zn and Cu) were analysed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Thermo Elemental Co. Iris Intrepid II XDL) with prior microwave acid sample digestion (8 mL of concentrated HNO_3 and 2 mL of H_2O_2 for 0.5 g DW), using a protocol previously described by Mora et al. (2010).

Data are expressed as nutrient uptake, defined as:

$$\text{Uptake}_{t25} = (\text{Nutrient amount})_{t0} - (\text{Nutrient amount})_{t25}$$

A theoretical nutrient amount was estimated for Zn-deficient plants considering their nutrient content and the difference of biomass between control and Zn-deficient plants.

$$\text{Uptake}_{-\text{Zn}-\text{theo}} = \left[(\text{Nutrient amount})_{\text{Control}} \times (MS_{-\text{Zn}}/MS_{\text{control}})\right] - (\text{Nutrient amount})_{t0}$$

2.3. Analysis of proteome

Protein extraction and 2–DE have been performed according to the protocol detailed in Desclos et al. (2008). Gels were stained using the silver–staining procedure described by Blum et al. (1987) and scanned with the ProXPRESS 2D proteomic imaging system