PP2A Methylation is Necessary for Broad Stress Tolerance

Maria T. Creighton

University of Stavanger

Dugassa Nemie-Feyissa

University of Stavanger

Nabeela Zaman

University of Stavanger

Sverre S. Johansen

University of Stavanger

Hege Dysjaland

University of Stavanger

Behzad Heidari

University of Stavanger

Cathrine Lillo (✉ cathrine.lillo@uis.no)

University of Stavanger

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Abstract

Background: LEUCINE CARBOXYL METHYL TRANSFERASE 1 (LCMT1) transfers a methyl group from the methyl donor S-adenosylmethionine (SAM) to the catalytic subunit of PROTEIN PHOSPHATASE 2A (PP2A). This post-translational modification of PP2A is manifested throughout eukaryotes from yeast to plants and animals. Although highly conserved, the importance of the methylation is poorly understood. Since Arabidopsis plants with knocked out *LCMT1* grow and develop fairly normally, we decided to search for conditions that may reveal the benefits of this regulation. We compared the effects of various stressful conditions on Arabidopsis wild type (WT) and a *lcmt1* mutant possessing only non-methylated PP2A.

Results: Seedlings were grown in Petri dishes for 5-12 days, or in rock wool and soil for up to 7 weeks. A significant increase in sodium concentration was found for *lcmt1* relative to WT, but this was not linked with stressful conditions. Plants were exposed to variable levels of the chelator EDTA, iron, zinc, aluminium, heat, and hydrogen peroxide. The *lcmt1* mutant was clearly more sensitive than WT to all the various stresses, as demonstrated by effects on seedling root growth and on shoots of rosette stage plants on rock wool. When omitting EDTA, expression of genes known as signature genes for iron deficiency, *FIT1*, *bHLH100*, *IMA1*, *IRT1* was strongly enhanced in *lcmt1*. Although an iron starvation response was induced, Fe homeostasis was apparently maintained by slowed growth in *lcmt1* and the Fe level related to tissue dry weight was not changed. Among genes induced in *lcmt1* were also the Zn induced gene *ZIF1*, and heat shock protein *HSP90-1*. Concentrations of non-iron transition metals, Cu, Mn and Zn, increased significantly in response to lack of EDTA for both *lcmt1* and WT tissue, and especially the growth of *lcmt1* was strongly hampered.

Conclusions: Presence of the *LCMT1* gene was necessary to cope efficiently with an imbalance in the micronutrients, heat stress, and oxidative stress. Methylation of PP2A appears important to ameliorate the toxic effects of metals present in unfavourable high concentrations as well as heat or oxidative stress. The experiments establish *LCMT1* as a key component in broad stress tolerance.

Background

PP2A (Protein Phosphatase 2A) is a highly conserved enzyme that removes phosphate from proteins in all eukaryotes, from yeast to animals and plants. In plants, PP2A is involved in the control of organ development, formative cell division, meiosis, various hormone signalling pathways, and responses to the environment (Durian, Rahikainen, Alegre, Brosché, & Kangasjärvi, 2016; Lillo et al., 2014; Michniewicz et al., 2007; Skottke, Yoon, Kieber, & DeLong, 2011; Tang et al., 2011; Waadt et al., 2015; Yoon, Ahn, & Pai, 2018; Yuan et al., 2018; Yue et al., 2016). The PP2A catalytic subunits are divided into two subgroups in eukaryotes. Impairment of all of the PP2A catalytic subunits within one of the two subgroups in Arabidopsis results in crippled seedlings, demonstrating the necessity for PP2A in growth and development (Ballesteros et al., 2013; Farkas, Dombradi, Miskei, Szabados, & Koncz, 2007). In addition to a catalytic subunit, the PP2A complex contains a scaffolding A and regulatory B subunit. In Arabidopsis

there are three different A and 17 different B subunits. The wide range of B subunits appear to be important for cellular localization and substrate specificity of the full complex (Farkas et al., 2007; Lillo et al., 2014).

PP2A itself is regulated by methylation of the C-terminal conserved leucine of the catalytic subunits. LCMT1 (LEUCINE CARBOXYL METHYL TRANSFERASE 1) transfers a methyl group from S-adenosyl-methionine to PP2A catalytic subunits. Loss of *LCMT1* resulted in almost complete loss of PP2A methylation in mammals (Lee, Wang, Sambo, Bunting, & Pallas, 2018), also, when *LCMT1* is impaired in Arabidopsis, PP2A is no longer methylated and is present only in its non-methylated form (Creighton et al., 2017; Ruan et al., 2018; Wu et al., 2011). In wild type Arabidopsis (WT), non-methylated PP2A generally makes up 20% of total PP2A (Creighton et al., 2017). It should be noticed that LCMT1 also methylates the close homologs of PP2A, i.e. PP4 and PP6 (Hwang, Lee, & Pallas, 2016). When the *LCMT1* gene is knocked out, a visible phenotype of *lcmt1* plants with more elongated rosette leaves is recognizable, but the mutant still grows vigorously and resembles WT plants (Chen, Hu, Zhu, Shen, & Zhang, 2014; Lillo et al., 2014; Wu et al., 2011; Zhang et al., 2019). However, under certain stressful conditions, for instance lack of chelator in the growth medium, we observed severe growth retardation and chlorosis. The methylation of a conserved motif in PP2A is conserved across different plant species, and across distant species like plants, fungi, and humans. This shows that methylation has indeed been favoured during evolution and indicates that some basic properties depend on this methylation. Plants needed to cope with a wide range of stressful environmental conditions throughout evolution and still do in various habitats, and methylation can be expected to be important at least under some of these conditions.

In yeast and mammalian cells the methylated form of PP2A appears to favour assembly with the B55/PR55 PP2A subunits (Gentry et al., 2005; Longin et al., 2007). In human, low activity of LCMT1 is associated with a low level of PP2A complexed with B55, resulting in highly phosphorylated tau protein, and likely aggravating Alzheimer's disease (Sontag et al., 2007). In mammals, *LCMT1* is also necessary for normal progression through mitosis (Lee & Pallas, 2007), and is a key player in hematopoiesis. (Lee et al., 2018). In mice, knockout of *LCMT1* has severe consequences, causing elevated apoptosis and embryonic lethality (Lee & Pallas, 2007). Clearly, LCMT1 was essential for the formation of blood components in mammals, but specific metabolic reactions dependent on LCMT1 were not studied or hypothesised, and the effects of methylation are still poorly understood. In mammalian cells, LCMT1 was found to be mainly localized to the cytoplasm, Golgi region and late endosomes (Longin et al., 2008). In Arabidopsis, LCMT1 was localized to the plasmalemma, cytosol and putative endosomes (Wu et al., 2011). An important effect of PP2A methylation by LCMT1 was suggested to be enhanced association of PP2A complexes with membranes, especially endosomes, and increased dephosphorylation of PP2A targets associated with membranes, for instance BRI1 (BRASINOSTEROID INSENSITIVE 1).

The micronutrients manganese, iron and zinc are needed for respiration and photosynthesis and as cofactors in a wide range of enzymes, including PP2A (Nishito et al., 1999). Micronutrients may need to be chelated to become available for uptake, or to avoid harmful reactions inside the cells. Although some

metals may be abundant in the soil, they are not always available for the plant. For example, under aerobic conditions iron is mostly present as Fe(III) which easily forms insoluble compounds in the environment. Plants secrete various substances to improve uptake of micronutrients, e.g. nicotianamine, phytochelators, organic acids and certain phenolics (Clemens, 2019; Seregin & Kozhevnikova, 2020). To facilitate uptake, the chelator EDTA is added to Murashige-Skoog (MS) medium (Murashige & Skoog, 1962) and Hoagland solution (Hoagland & Arnon, 1950). In Arabidopsis, Fe(III) is first reduced by FRO2 (FERRIC REDUCTION OXIDASE/REDUCTASE 2) at the root surface, and then the main transporter IRT1 (IRON-REGULATED TRANSPORTER 1) brings Fe(II) into the cell. IRT1 also enables transport of zinc, manganese, copper, cadmium and cobalt ions. Various chaperons, storage proteins and low-molecular-weight ligands assure ion homeostasis inside the cells (de Abreu-Neto, Turchetto-Zolet, de Oliveira, Bodanese Zanettini, & Margis-Pinheiro, 2013; Fukao et al., 2011; Seregin & Kozhevnikova, 2020). Since micronutrient homeostasis is essential for all eukaryotes, and we had observed the importance of EDTA for growth of the *lcm1* mutant in early experiments, we decided to test if stress by non-optimal levels of iron and zinc would be aggravated in the *lcm1* mutant. Another metal, aluminium, is a common constraint to crop growth, especially in acidic soil which makes up about one third of the ice-free land area of the world (Horst, Wang, & Eticha, 2010), and the addition of abundant aluminium was tested for plants grown in rock wool. Reactive oxygen species (ROS) are mainly products of basic metabolism and generated during respiration in the mitochondria and reactions linked with photosynthesis in the chloroplasts and peroxisomes. At low levels, ROS are important in signalling pathways, but excess ROS can damage cellular components and lead to destruction of the cell unless scavenging mechanism are active. H₂O₂ is normally generated in peroxisomes during photorespiration and in microbodies during lipid catabolism as a side-product of fatty acid oxidation and can diffuse between different compartments of the cell. ROS are also common intermediates resulting from many different types of stress, including metal stress and heat stress (Bheri & Pandey, 2019; Mittler, Vanderauwera, Gollery, & Van Breusegem, 2004; Ohama, Sato, Shinozaki, & Yamaguchi-Shinozaki, 2017). Responses to heat stress are found in all types of organisms, and heat shock transcription factors are conserved among eukaryotes. Heat shock transcription factors can be rapidly induced and lead to induction of other protective components, especially HEAT SHOCK PROTEINS (HSPs) (Ohama et al., 2017; Samakovli et al., 2020). Temperature fluctuates throughout the day and night, and the global trend of increasing temperature will also have a great influence on plants. Temperature strongly influences catalytic reactions, including enzymatic reactions, and high or low temperatures may disrupt the balance in metabolism. High temperature will also increase membranes fluidity and affect various compartments in the cell. HSPs are necessary for tolerance to heat and other stresses, and HSPs are ubiquitous in animals, plants and microorganisms (Samakovli et al., 2020). HSP90s are chaperons important for the response to both heat and various environmental signals. HSP90s are known to activate several kinases, and are also important for development, like stomatal formation (Mazaira, Daneri-Becerra, Zgajnar, Lotufo, & Galigniana, 2018; Samakovli et al., 2020).

Although a phenotype is visible with more narrow leaves and early flowering, the *lcm1* knockout mutant shows growth and development similar to wild type (Creighton et al., 2017). This raises the question why

LCMT and PP2A methylation is conserved also in plants. Under what conditions may LCMT be beneficial to plants? In order to elucidate this question, we set off to study the phenotype of Arabidopsis with mutated *lcmt1* under various stress conditions, especially stresses caused by disturbing the nutrient balance, oxidative stress, and heat stress.

Material And Methods

Seedlings for observing phenotype

Seeds of WT and *lcmt1* (At1g02100, SALK_079466, (Alonso et al., 2003)) obtained from the European Arabidopsis Stock Centre in Nottingham UK, and genotyped as described in Creighton et al. (Creighton et al., 2017). Plants were grown in certified chambers for gene modified plants and handled accordingly, and no permissions were necessary to collect such samples. Seeds were surface sterilized by calcium-hypochlorite/ethanol and sown in Petri dishes containing ½ MS medium (Murashige & Skoog, 1962) salts and 1% sucrose. The plates were placed in the dark at 4°C for three days to ensure even germination and thereafter placed at 22°C in 16 h light and 8 h dark cycles. After 5 d at 22°C, seedlings were transferred to new plates, plates with control MS medium and plates with medium lacking EDTA or containing various compounds to be tested. Roots were measured using imageJ (<https://imagej.nih.gov/ij/download.html>).

Rosette stage plants

Plants were cultivated in soil for three weeks in a 16 h light/8 h dark regimen and given only water, then plants were moved to rock wool and grown in short days 8 h light /16 h dark (to avoid transition to flowering) cycles with Hoagland solution containing FeCl₃ or FeSO₄ as iron source, and with or without EDTA.

RNAseq

After sowing on ½ MS medium salts and 1% sucrose and omitting EDTA, the Petri dishes were placed at 4°C for three days as usual followed by 5 days at 22°C before harvesting. At this stage WT and *lcmt1* seedlings still looked similar and were considered suitable for harvest and comparison of gene expression. RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA, US), and treated with RNAase free (Qiagen)DNase. Eurofins Genomics Europe Sequencing GmbH (Konstanz, Germany) carried out the RNA-seq, three samples for both *lcmt1* and WT, averages of the three samples are presented in supplementary Table S2.

Element content

Plant tissue was powdered in liquid nitrogen using a mortar and pestle, then dried for 48 h at 60°C, then 3 days in a desiccator before analysed by ICP (Inductive coupled plasma mass spectrometry) at NIBIO (Norsk institutt for bioøkonomi, Kjemisk laboratorier), Ås, Norway. For seedlings around 300 shoots were

harvested for each sample to make the minimum 100 mg dry weight. For rosette stage plants about 20 rosettes were used for one sample

Results

Visual effects of excluding EDTA from the growth medium

In early experiments, we observed negative effects on seedlings and rosette plants when omitting the EDTA chelator from the growth medium, and the effects were strikingly stronger for *lcmt1* as compared with WT (Fig. 1). After germination of seedlings on complete MS medium, seedlings were transferred to fresh MS medium or MS medium lacking EDTA. This resulted in chlorosis of shoots and impairment of root growth (Fig. 1). Apparently *lcmt1* was less resistant to the stress exerted by lack of EDTA, with strongly inhibited root growth (Fig. 1b). To explore effects of stressful conditions on a later developmental stage, plants were cultivated in soil for three weeks, then moved to rock wool with Hoagland solution with or without EDTA and containing FeCl_3 or FeSO_4 as iron source (Fig. 1c-f). Both WT and *lcmt1* grew well with Hoagland prepared with EDTA in combination with FeCl_3 or FeSO_4 (Fig. 1c, e). When plants were given FeCl_3 without EDTA both WT and *lcmt1* became chlorotic (Fig. 1d), and growth of *lcmt1* was strongly hampered. When plants were given FeSO_4 as iron source without a chelator (Fig. 1f), also both WT and *lcmt1* became chlorotic, and the stress symptoms were again more severe for *lcmt1* (Fig. 1d). Clearly, as for seedlings, omitting EDTA stressed the plants and *lcmt1* was more affected than WT.

Iron-EDTA

To further get insight into different stress responses of *lcmt1* seedlings, the concentration of Fe-EDTA (FeNa_2EDTA) was varied from 0 to 500 mM in the agar medium (Fig. 2, Supplemental Fig. S1). Optimal seedling growth was observed at 25 and 50 mM (concentrations in half-strength and regular MS medium) for both WT and *lcmt1*. Shoots of *lcmt1* generally had lower fresh weight than WT. At standard concentration (25 mM Fe-EDTA) fresh weight of *lcmt1* shoots was 70% of WT fresh weight. To be able to compare changes in *lcmt1* and WT related to the different treatments, all data was standardized to fresh weight and root elongation at 25 mM set to one (Fig. 2). After growth for six days with different iron concentrations, shoot weight of WT seedlings was lower by approximately 45 and 70% when exposed to 350 and 500 mM Fe-EDTA, respectively, but no significant difference in decreases was seen for *lcmt1* as compared with WT. However, from 200 mM and higher Fe-EDTA there was a negative effect on root elongation in *lcmt1* as compared with WT. At 500 mM, roots of both WT and *lcmt1* stopped growing (Supplemental Fig. S1). The results showed that high concentrations of Fe-EDTA impaired root growth in *lcmt1* more strongly than in WT (Fig. 2b). Interestingly, at zero Fe-EDTA root growth was also more strongly affected in *lcmt1* than in WT.

Zinc and other micronutrients

The effects of excluding EDTA from the agar medium in the presence and absence of various micronutrients were tested and showed that fresh weight of shoots was severely reduced in response to lack of EDTA or lack of all micronutrients (Fe, Zn, B, Mn, Mo, Cu, Co, I) (Fig. 3). Addition of 16 mM zinc (standard concentration) led to increased fresh weight, but only when comparing media containing EDTA. A ten times higher, 160 mM, concentration of zinc did not have positive effects. In conclusion, when avoiding the micronutrients, fresh weight of both WT and *lcmt1* was more than 50% reduced, but there were no obvious differences between the responses of *lcmt1* and WT regarding fresh weight (Fig 3a). On the other hand, growth of roots was more strongly impaired in *lcmt1* compared with WT in all media lacking micronutrients and/or EDTA (Fig. 3b).

Aluminium

Shoot fresh weight of seedlings grown on agar decreased when $\text{Al}_2(\text{SO}_4)_3$ was added in concentrations of 0.4 or 0.6 mM, but with no significant difference in response between WT and *lcmt1* (Fig. 4a). Roots of *lcmt1* grew significantly less in the presence at concentrations 0.2, 0.4, and 0.6 mM as compared with WT (Fig. 4b, Fig. S2.).

Heat stress

Seedlings were exposed to 37°C for 6-48 h and compared with non-exposed controls. After 18 h or longer time at 37°C the seedlings gained less shoot fresh weight during the subsequent 6 days, but there was no difference between WT and *lcmt1* (Fig. 5a, Fig. S3). Negative effects on root growth were observed after 12 h of heat stress (Fig. 5b). After 24, 36 and 48 h, the effects on root growth were stronger for *lcmt1* than for WT (Fig. 5b, Fig. S3).

Heat stress was also tested with rosette plants grown in soil for 5 weeks. Plants were exposed to heat (37°C) for 0, 8, 18, 24 h, then grown at 22°C. Pictures were taken i) immediately after treatment, ii) after 1 week and iii) after 2 weeks (Fig.6, Fig. S4). Both WT and *lcmt1* grew well after 8 or 18 h heat treatment (Fig S4). After 24 h heat treatment, inspecting the plants after 1 week of recovery showed more green leaves for WT in three repeats (Fig. 6, Fig. S4), however, after the second week all plants died indicating that the shoot meristem was impaired in both *lcmt1* and WT.

ROS are common metabolic intermediates and a result of many different types of stress, including metal stress and heat stress. Experiments confirmed that also ROS, in the form of H_2O_2 added to the agar medium at 0.5-2.5 mM), resulted in stronger inhibition of root growth in *lcmt1* than in WT (Supplemental Fig S5).

Element analysis

Element analysis was performed for shoots of seedlings grown in agar in Petri dishes without or with EDTA for 12 days, and for shoots of 6-week-old rosette stage plants grown first on soil for three weeks, then on rock wool for 3 weeks without or with EDTA, or without and with iron. When comparing seedlings

of *lcmt1* and WT, the differences in element contents were generally less than 10% or too variable to become significant (Table 1 and supplemental Table S1). When comparing seedlings grown without or with EDTA (pooling WT and *lcmt1*) there was a clear difference for zinc content; seedlings grown without EDTA having 48% more zinc than seedlings with EDTA. For iron and sulphur, when omitting EDTA, there was a 12% decrease or increase, respectively (Table 1).

For 6-week-old plants on rock wool, as for seedlings, significant differences between element contents in rosettes of *lcmt1* and WT were generally not found, but with one interesting exception; sodium content was elevated by 64% in *lcmt1*. The elevated sodium level for *lcmt1* persisted also for plants grown without iron, on soil, or with extra aluminium (Supplemental Table S1). As for seedlings, when grown without EDTA, rosette plants had increased levels of zinc (increased by 89%) (Table 1). For the rosette stage plants, other micronutrients (Cu, Mn, Mo, Ni) also showed elevated levels in the absence of EDTA. From the elevated elements, especially Cu, Mn and Zn were present at high concentrations 40, 185 and 59 mg/g DW, respectively (data based on Table S1). Such high metal levels may have influenced several metabolic reactions and led to impaired growth and chlorosis (Fig. 1d, f). Other micronutrients responding significantly when omitting EDTA were molybdenum and nickel, but these represented only 8 and 3 mg/g DW, and would hardly be harmful. Apparently, in the absence and presence of EDTA, homeostasis mechanisms kept iron constant (Table 1, Table S1), but growth was impaired. When iron was excluded from the nutrient solution (but EDTA was kept), the level of iron in rosettes decreased by 41% (Table 1, bottom row). Altogether the element analysis showed clear effects of excluding the chelator in the nutrient solution, especially by increased levels of the micronutrients Cu, Mn and Zn, however, significant differences related to the *lcmt1* mutation was found only for sodium, with increased levels in *lcmt1* plants.

Table 1. Relative element contents in shoots of seedlings on agar or shoots of plants on rock wool. Seedlings: seeds were sown in Petri dishes, grown for 12 days in MS medium without or with EDTA. Rosettes: plants were first grown for 3 weeks in soil, then transferred to rock wool for 3 more weeks with different nutrient solutions. The Hoagland solution was made without or with EDTA, or without and with iron. Data are given for i) an element in *lcmt1* seedlings as percentage of that element in WT seedlings; ii) an element in seedlings grown in the absence of EDTA as percentage of that element in seedlings grown in the presence of EDTA; iii) an element in *lcmt1* rosettes as percentage of that element in WT rosettes; iv) an element in rosettes grown in the absence of EDTA as percentage of that element in rosettes grown in the presence of EDTA; v) an element in rosettes grown in the absence of iron as percentage of that element in rosettes grown with iron. P values are given for all comparisons. Percentages that were significantly different from 100 with $p < 0.05$ by TTEST are high-lighted in red,

	Mineral content, one type of sample as percentage of other type of samples, %										
	Fe	B	Ca	Cu	Mn	Mo	Na	Ni	P	S	Zn
Sample type											
i) Seedlings lcmt1/WT	101	106	104	124	106	94	108	98	91	107	110
p (n=4)	0.94	0.20	0.24	0.50	0.31	0.02	0.08	0.36	0.03	0.22	0.63
ii) Seedlings EDTA no/yes	88	97	97	126	93	101	98	102	107	112	148
p (n=4)	0.02	0.58	0.45	0.46	0.22	0.71	0.74	0.36	0.18	0.01	0.01
iii) Rosettes lcmt1/WT	125	74	94	96	90	78	164	250	124	116	115
p (n=8)	0.20	0.05	0.39	0.93	0.75	0.29	0.001	0.03	0.38	0.49	0.66
iv) Rosettes EDTA no/yes	103	86	117	427	251	173	85	244	205	198	189
p (n=8)	0.92	0.35	0.010	9.3E-5	0.002	0.01	0.3620	0.03	0.0006	2.6E-5	0.03
v) Rosettes iron no/yes	59	121	97	84	140	207	113	80	116	110	89
p (n=4)	0.005	0.0001	0.60	0.02	0.01	0.008	0.36	0.36	0.16	0.52	0.25

RNAseq

After sowing and three days at 4°C, WT and *lcmt1* seedlings were germinated and grown under stressful conditions in Petri dishes by omitting EDTA from the growth medium during 5 days at 22°C, then harvesting. WT and *lcmt1* seedlings still looked very similar and were considered suitable for comparison of changes in gene expression caused by lack of EDTA. Some seedlings were left to grow for another week and confirmed the strong growth inhibition on *lcmt1* when EDTA was omitted for some more days (as in Fig. 1b). Almost 3 000 genes showed significantly different expression levels in *lcmt1* and WT, 1528 genes were up-regulated and 1366 genes were down-regulated in *lcmt1* in comparison with WT. When the limit was set to 2x different expression levels, 520 genes were up-regulated and 653 genes were down-regulated in *lcmt1* as compared with WT. Several genes (155) were more than 5x downregulated in *lcmt1* (Table 2, Supplemental Table S2 with original data).

Table 2. Number of genes differentially expressed in *lcmt1* and

WT when grown on ½ MS medium lacking EDTA.

	Number of genes
Genes significant differently expressed	2894
Genes up-regulated in <i>lcmt1</i>	1528
Genes down-regulated in <i>lcmt1</i>	1366
Genes > 5x different in <i>lcmt1</i> and WT	194
Genes > 5x up-regulated in <i>lcmt1</i>	39
Genes > 5x down-regulated in <i>lcmt1</i>	155
Genes > 2x different in <i>lcmt1</i> and WT	1173
Genes > 2x up-regulated in <i>lcmt1</i>	520
Genes > 2x down-regulated in <i>lcmt1</i>	653

GO (Gene Ontology) enrichment was analysed by help of Panther (Mi et al., 2021) which divides genes into groups belonging to three categories; Biological processes, Molecular function, and Cellular components. Examples of enriched ontologies for genes 2x differently expressed are presented in Table 3. Many enriched groups extensively overlapped, therefore, not all groups are listed. Complete lists are presented in the Supplemental material (Supplemental Tables S3-S8). Genes up-regulated in *lcmt1* were enriched in iron homeostasis genes, synthesis of cutin and various stress-annotated genes. Iron homeostasis genes were further inspected (Fig. 7). These genes included five *BHLH* transcription factors and the *BRUTUS E3* ligase involved in regulation of iron homeostasis regulation, two genes directly involved in uptake of iron from the environment i.e. *IRT1* and *FRO2*, the vacuolar localized metal transporters *FRD3* and *NRAMP4*, and the oligopeptide transporter gene encoding a phloem specific iron transporter gene *OPT3*. We manually added three *IMA* genes (*IRONMAN1,2,6*). Other *IMA* genes, *IMA 3* and *4* were also 4-5 times higher expressed in *lcmt1*, but are not included in the figure. *IMA* genes were recently shown to be crucial for regulation of iron transport and homeostasis (Grillet, Lan, Li, Mokkaapati, & Schmidt, 2018). We also added genes involved in chelating or storage of iron. Three *NAS* genes (1, 2, 4) encoding enzymes that synthesise the chelator nicotianamine were upregulated, whereas the iron storage protein *FER1* was downregulated in *lcmt1*, indicating less storage capacity. The *PP2AA3* gene, used as a control, was constant. Many stress related genes were differentially expressed in *lcmt1* and WT, they were either up (97) or down (128) regulated in *lcmt1*. Transporter genes were also differentially expressed, both up (41) and down regulated (128) in *lcmt1* (Table 3). Genes involved in photosynthesis, i.e. light harvesting, were strikingly downregulated in *lcmt1*. Also, genes encoding hem-binding proteins many of which are cytochromes, were down-regulated in *lcmt1*. The ZINC-INDUCED FACILITATOR (*ZIF1*) was expressed at a high level and 2.9 times higher in *lcmt1* than in WT seedlings (Table S2). *ZIF1* is important for transportation of nicotianamine and Zn into the vacuole and critical for both Zn and Fe

homeostasis (Haydon et al., 2012). The heat shock protein HSP90-1 known to be involved in many different types of stresses was 2.2 times higher in *lcm1* than WT (Table S2).

Table 3. GO (Gene ontology) enrichment. Statistical over/under representation (powered by Panther, https://www.arabidopsis.org/tools/go_term_enrichment.jsp). Genes expressed at a level at least 2 times higher or lower in *lcm1* as compared with WT were included in the analysis (Details are shown in Table S3-S8).

	number of genes	fold enriched
Biological processes. 2x higher in <i>lcmt1</i>		
iron ion homeostasis	10	12.80
cutin biosynthesis	5	20.62
response to water deprivation	18	3.72
response to osmotic stress	30	4.22
response to temperature stimulus	25	2.97
response to stress	97	3.31
Molecular function. 2x higher in <i>lcmt1</i>		
transmembrane transporter activity	41	2.47
Cellular component. 2x higher in <i>lcmt1</i>		none
Biological processes. 2x lower in <i>lcmt1</i>		
Photosynthesis, light harvesting in photosystem 1	8	20.30
Response to light stimulus	43	3.68
Defence response to other organisms	58	4.38
Response to stress	128	2.50
Response to ethylene	13	5.07
Response to salicylic acid	19	7.66
Response to oxygen levels	16	3.66
Response to temperature	30	2.92
Response to fungus	27	4.70
Response to bacteria	43	5.11
Molecular function. 2x lower in <i>lcmt1</i>		
Anion transmembrane transporter activity	24	2.72
Chlorophyll binding	7	15.22
Heme binding	23	3.76

oxidoreductase activity	49	2.12
Cellular component. 2x lower in <i>lcmt1</i>		
plastoglobule	9	7.21

Discussion

The various stressful conditions tested on Arabidopsis seedlings in Petri dishes all resulted in stronger inhibition of primary root growth in *lcmt1* than in WT. WT also showed impaired root growth in response to the different treatments, but *lcmt1* was always more susceptible to the stresses. These stresses were: lack of chelator, elevated levels of iron, zinc and aluminum, heat, and oxidative stress. Seedling shoot weight was influenced by all stresses tested, and the decrease in shoot fresh weight was the same in *lcmt1* and WT. This may be explained by the relatively short time period for the seedlings' exposure to stress (6 days) because plants in rock wool under stress for a longer time (three weeks) showed clear differences between shoot growth of *lcmt1* and WT, with *lcmt1* growth being much stronger impaired (Figs. 1). The fact that *lcmt1* showed decreased primary root growth, regardless of the type of stress imposed, indicates that a basic function of PP2A methylation is involved.

Elements

Total element analysis revealed that one element, sodium, accumulated differently in rosette stage plants of *lcmt1* and WT, with higher levels in *lcmt1* (plus 64 %), and this was reproducible throughout different experiments regardless of the presence of chelator or iron, or growth in rock wool or soil (Table 1 and Supplemental Table S1). Other elements did not vary systematically between WT and *lcmt1*. Omitting EDTA for three weeks had a pronounced effect on element contents for both genotypes, with high accumulation, especially of the micronutrients copper, manganese, and zinc (Table 1 and Table S1). Interestingly, the level of iron was not much influenced by omitting EDTA, homeostasis was apparently upheld for this element (but growth impaired). The concentration of copper, manganese and zinc, all together, made up a concentration two times that of iron in plants growing without EDTA, but in plants with EDTA these elements summed up to a concentration lower than iron (by 17%). The accumulation of copper, manganese and zinc may have given an ion imbalance and replaced iron in important proteins and cofactors leading to impaired plant growth. Apparently, WT, with methylated PP2A, is better to cope with this imbalance than *lcmt1*.

Native PP2A isolated from mammals contains iron and zinc in its active site, while recombinant PP2A catalytic subunit was found to also have manganese. Furthermore, manganese binding was associated with binding of the PP2A chaperon PTPA. During in situ activation of PP2A in mammals, binding of the PTPA chaperon and manganese apparently led to PP2A being active not only towards phosphorylated Ser/Thr residues, but also towards phosphorylated tyrosyl residues. After binding of the PTPA chaperon, LCMT1 methylates PP2A, which leads to release of the chaperon causing specificity of PP2A towards phosphorylated Ser/Thr residues only (Guo et al., 2014). Assuming the same activation mechanism

applies to plants; since PP2A is not methylated in the *lcmt1* mutant, PP2A may be more influenced by an imbalance of the micronutrients and specificity towards substrates may decrease because of tighter binding of the PTPA chaperon. Our hypothesis is that high levels of manganese in the plant tissue, as was a result of omitting the chelator EDTA, may be especially damaging in the *lcmt1* mutant by promoting less specificity of PP2A.

Transporters and protein cycling

Many of the genes involved in iron homeostasis were highly induced in *lcmt1* seedlings, and appeared as an enriched gene ontology group, about 13 times enriched (Table 3, Fig. 7). The *lcmt1* seedlings responded as if iron-depleted, however, the element analysis did not reveal lack of iron. The iron transporter IRT1 is present in the plasma membrane as a complex with FRO2 and the proton pump AHA2. IRT1 itself is constantly cycling between the plasma membrane and endosomes. In response to high levels of non-iron metals like manganese and zinc, IRT1 is phosphorylated, which then leads to dissociation of the complex, and endocytosis of IRT1 (Barberon et al., 2014; Dubeaux, Neveu, Zelazny, & Vert, 2018; Martín-Barranco, Spielmann, Dubeaux, Vert, & Zelazny, 2020). The phosphorylation status of IRT1 is the result of a balance between phosphorylation by a protein kinase (CIPK23), and an unknown protein phosphatase. If the methylated form of PP2A is of importance for dephosphorylation of IRT1, this could be a way that *LCMT1* impinges on the IRT1 and iron homeostasis. Methylated PP2A has previously been found to be more frequently localized to endosomes than non-methylated PP2A (Wu et al., 2011). Possibly, lack of methylated PP2A, as in the *lcmt1* mutant, could lead to unbalanced IRT1 endo/exocytosis, but these mechanisms remain unclear. The gene expression analysis pointed to a range of transmembrane transporters differentially expressed in *lcmt1* and WT, and points to *lcmt1* being of importance for membrane functions (Table 3, Table S7).

Sodium

Element levels were very similar in *lcmt1* and WT except for sodium, which was increased by 64% in samples of rosette shoots of *lcmt1* relative to WT (Table 1). The NHX1 and NHX2 are the main contributors to transport of sodium and potassium into vacuoles in Arabidopsis and essential for ion balance in plants (Bassil, Zhang, Gong, Tajima, & Blumwald, 2019). The *NHX1* transcript level was more than 10 times higher than the other vacuolar *NHX* transcripts, indicating its importance for ion homeostasis in the present experiments. *NHX1* was significantly upregulated (by factor 1.7) in *lcmt1* relative to WT as revealed in the RNAseq data (Table S1) and pointing to the possibility that the *lcmt1* mutation inferred with sodium balance. However, the accumulation of sodium in *lcmt1* may possibly also be linked with other genes, and the transporters of sodium are still not fully identified (Bassil et al., 2019).

Conclusion

The mutant *lcmt1* was always more susceptible to various stress treatments than WT. The methylation of PP2A apparently is of fundamental value for coping with stress and appears to be important for tolerating an imbalance in the micronutrients.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: All data used are available in the article and supporting information.

Competing interests: The authors declare that they have no competing interests.

Authors' contributions: M.T.C., B.H. and C.L. conceived and designed the experimental plan

M.T.C., D. N-F, N.Z. S.S.J., H.D. and B.H. performed the experiments

C.L. and M.T.C. drafted the manuscript.

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Figures

Figure 1

WT and *lcmt1* seedlings and rosette stage plants grown on agar medium or rock wool. Seedlings: Seeds were germinated for 5 days on complete MS medium, and length of the roots at transfer to different media was marked (black dots). Seedlings were then grown for 7 days on half strength MS medium with a) complete MS and b) MS without the EDTA chelator. Rosette stage plants: After growth in soil for three weeks, plants were transferred to rock wool for another three weeks and watered with Hoagland solution with or without EDTA and with two different iron sources; c) FeCl₃, with EDTA; d) FeCl₃, no chelator; e) FeSO₄, with EDTA; f) FeSO₄, no chelator.

Figure 2

Comparison of WT and *lcmt1* seedling growth on agar medium with different concentrations of Fe-EDTA (FeNa₂EDTA). a) Shoot relative fresh weight; b) Primary root relative elongation. After germination on the standard half strength MS medium for 5 days, seedlings were transferred to the media with different Fe-EDTA concentrations and grown for 6 days. Data were normalized to the values at 25 mM (control/standard medium). Mean control values were 8.3 mg and 5.8 mg fresh weight per seedling for

WT and lcmt1, respectively, and 75 mm elongation of primary roots for both WT and lcmt1. Data are averages of 30 seedlings for each treatment and genotype, SE is given, a star indicates significant difference between WT and lcmt1 by TTEST, for $p < 0.05$.

Figure 3

Comparison of WT and lcmt1 growth on agar medium with or without micronutrients and EDTA. a) Shoot relative fresh weight; b) Primary root relative elongation. After germination on the standard half strength

MS medium for 5 days, seedlings were transferred to the different media, half MS medium with and without EDTA, media lacking micronutrient with or without EDTA (no E), and media with addition of Zn at 16 mM or 160 mM but with no other micronutrient. Data were normalized to the values at 25 mM (control medium with EDTA) set to one. Data are based on 45 seedlings for each treatment and plant type, SE is given as vertical bars. A star indicates significant difference between WT and *lcmt1*, $p < 0.05$ by TTEST.

Figure 4

Comparison of WT and *lcmt1* seedling growth on agar medium with different concentrations of aluminium. a) Shoot relative fresh weight; b) Primary root relative elongation. After germination on the standard half strength MS medium for 5 days, seedlings were transferred to the media with different concentrations of $\text{Al}_2(\text{SO}_4)_3$: 0, 0.2, 0.4 and 0.6 mM and grown for another 6 days. Data are based on 45 seedlings for each treatment and plant type, SE is given as vertical bars. A star indicates significant difference between WT and *lcmt1*, $p < 0.05$ by TTEST.

Figure 5

Effects of heat stress on WT and *lcmt1* seedlings. a) Shoot relative fresh weight; b) Primary root relative elongation. After germination on the standard half strength MS medium for 5 days, seedlings were transferred to fresh medium and exposed to 37°C for 0, 6, 12, 18, 24, 36 and 48 h. After the heat treatment, plates were placed back to 16 h light/8 h dark cycles at 22°C for 6 days before assessment. Data are based on 45 seedlings for each treatment and plant type, SE is given. A star indicates significant difference between WT and *lcmt1*, $p < 0.05$ by TTEST.

Figure 6

Effects of heat stress on WT and *lcmt1* rosette plants. Plants were grown in soil for 5 weeks, then treated with heat (37°C) for 24 h, and placed back at 22°C. Pictures were taken immediately after treatment, after 1 week and 2 weeks (more repeats are given in Fig. S4).

Figure 7

Expression of genes involved in iron homeostasis. Expression levels in *lcmt1* are given relative to expression in WT. Seedlings were grown on ½ MS medium without EDTA for 5 days before harvesting whole seedling for RNAseq. The genes included are iron homeostasis regulator genes (five bHLH transcription factors, BTS and IMA genes), genes directly involved in uptake from the environment (IRT1, FRO2), genes encoding vacuolar metal transporters (FRD3, NRAMP4), gene encoding a phloem iron transporter OPT3, three nicotianamine synthesis (NAS) genes, a gene encoding the iron storage protein FER1, and the reference gene PP2AA3 (for more details see Table S2 RNAseq).

Supplementary Files

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