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Loss of *LEUCINE CARBOXYL METHYLTRANSFERASE 1* interferes with metal homeostasis in Arabidopsis and enhances susceptibility to environmental stresses

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ABSTRACT

The biochemical function of LEUCINE CARBOXYL METHYLTRANSFERASE 1 (LCMT1) is to transfer a methyl group from the methyl donor S-adenosylmethionine (SAM) to the catalytic subunits of PROTEIN PHOSPHATASE 2A (PP2Ac), PP4 and PP6. This post-translational modification by LCMT1 is found throughout eukaryotes from yeast to animals and plants, indicating that its function is essential. However, Arabidopsis with knocked out *LCMT1* still grows and develops almost normally, at least under optimal growth conditions. We therefore proposed that the presence of *LCMT1* would be important under non-optimal growth conditions and favoured plant survival during evolution. To shed light on the physiological functions of plant *LCMT1*, phenotypes of the *lcmt1* mutant and wild type Arabidopsis were compared under various conditions including exposure to heavy metals, variable chelator concentrations, and increased temperature. The *lcmt1* mutant was found to be more susceptible to these environmental changes than wild type and resulted in poor growth of seedlings and rosette stage plants. Element analysis of rosette stage plants mainly showed a difference between the *lcmt1* mutant and wild type regarding concentrations of sodium and boron, two-fold up or halved, respectively. In both *lcmt1* and wild type, lack of EDTA in the growth medium resulted in enhanced concentration of copper, manganese, zinc and sulphur, and especially *lcmt1* growth was hampered by these conditions. The altered phenotype in response to stress, the element and mRNA transcript analysis substantiate that *LCMT1* has an important role in metal homeostasis and show that functional *LCMT1* is necessary to prevent damages from heat, heavy metals or lack of chelator.

1. Introduction

LEUCINE CARBOXYL METHYLTRANSFERASE 1 (LCMT1) transfers a methyl group from S-adenosylmethionine to the carboxyl group of the C-terminal end of the PROTEIN PHOSPHATASE 2A (PP2A) catalytic subunit (PP2Ac). This methylation is important for activity and cellular localization of the full PP2A complex which is a major protein phosphatase in eukaryotes (Elgenaidi and Spiers, 2019). PP2Ac and its key regulatory proteins, like LCMT1, are highly conserved in eukaryotes (Bheri and Pandey, 2019; Booker and DeLong, 2017; Farkas et al., 2007; Lillo et al., 2014). The C-terminal motif becoming methylated, TPDYFL, is conserved in all organisms, and same or similar in the PP2A-like enzymes PP4 and PP6, which also can be methylated by LCMT1 (Hwang et al., 2016; Nasa and Kettenbach, 2020). The crystal structures of yeast and mammalian LCMT1 (Leulliot et al., 2004; Stanevich et al., 2011) showed that the active site of LCMT1 binds to the last six amino acids at the C-terminal end of PP2Ac, and additionally, the catalytic site of PP2A also makes extensive interactions with LCMT1. Since the catalytic sites of PP2Ac, PP4 and PP6 are highly conserved, this makes LCMT1 highly specific for these substrates (Nasa and Kettenbach, 2020). LCMT1 has been studied most intensively in yeast and mammals where it was found to be necessary for normal progression through mitosis (Lee and Pallas, 2007). In mammals, LCMT1 was necessary for hematopoiesis (Lee et al., 2018), and knockout of *LCMT1* caused embryonic lethality (Lee and Pallas, 2007). LCMT1 exerts its effects through promoting increased levels of methylated PP2Ac and PP2A-like enzymes (PP4, PP6). Although this has not been studied in plants, methylation of yeast or mammalian PP2Ac is known to favour formation of complexes with certain regulatory subunits (Bα/PR55/B55) and also leads to an increase in total PP2A activity (Elgenaidi and Spiers, 2019; Gentry et al., 2005;

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Longin et al., 2007). PP2Ac methylation is reported to be important for association with various membranes, i.e. plasma membrane microdomains in mammalian cells (Sontag et al., 2013) and microsomal fractions in plants (Wu et al., 2011). In mammals, as well as plants, the methylated form of PP2Ac is dominating, and represents 70–90% of the total PP2Ac present in cells (Creighton et al., 2017; Nasa and Kettenbach, 2020; Yu et al., 2001). In the Arabidopsis *lcmt1* mutant used in this and previous work, the level of total PP2Ac protein was the same or slightly lowered (Chen et al., 2014; Creighton et al., 2017), but the level of non-methylated PP2Ac was strongly elevated in the *lcmt1* mutant, i.e. six times higher than in wild type (WT) (Creighton et al., 2017). The strong conservation of the methylatable PP2Ac motif across species and detrimental effects of loss of this methylation in mammals imply that important physiological functions are expected to rely on a functional *LCMT1* also in other multicellular eukaryotes. Plants needed to cope with a wide range of stressful environmental conditions throughout evolution and still do in various habitats. Methylation of PP2Ac, PP4 and PP6 can be expected to favour survival at least under some of these conditions. Although a phenotype is visible with more narrow leaves and early flowering, the *lcmt1* mutant shows growth and development similar to wild type under optimal growth conditions (Creighton et al., 2017). This raised the question why *LCMT1* is conserved also in plants, and under what conditions *LCMT1* may be beneficial to plants. In order to elucidate these questions, we studied the phenotype of the *lcmt1* mutant under various stressful conditions and identified conditions requiring *LCMT1* for optimal growth.

2. Materials and methods

2.1. Seedlings

Seeds of WT and *lcmt1* (At1g02100, SALK_079466) (Alonso et al., 2003) were obtained from the European Arabidopsis Stock Centre in Nottingham UK, and genotyped as described in Creighton et al. (2017). Seeds were surface sterilized by calcium-hypochlorite/ethanol and sown in Petri dishes containing ½ MS salts (Murashige and Skoog, 1962) and 1% sucrose. The Petri dishes 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/8 h dark cycles. After 5 days at 22 °C, seedlings were transferred to new plates with i) control ½ MS medium with 1% sucrose, ii) medium lacking EDTA, or iii) containing various concentrations of Fe, Zn, or Al. After 6 days, roots were measured using ImageJ (https://imagej.nih.gov/ij/download.html) and fresh weight of shoots was measured.

2.2. Rosette stage plants

Plants were cultivated at 22 °C in soil for three weeks in a 16 h light/8 h dark regimen and given only water, then carefully removed, and roots were soaked in water to remove soil before transferred to rock wool with modified Hoagland solutions (Hoagland and Arnon, 1950). At this stage plants were moved to short days, 8 h light/16 h dark cycles (to avoid transition to flowering). For the heat treatment experiments, plants were grown continuously in soil for 5 weeks, placed at 37 °C for 8 or 24 h, and then placed back at 22 °C for observation.

2.3. Element content

Seedling shoots: plants were grown in Petri dishes for 12 days in ½ MS medium with 1% sucrose, without or with EDTA. Around 300 shoots were harvested for each sample to make the minimum 100 mg dry weight. Rosettes: plants were grown for 3 weeks in soil, then transferred to rock wool for 3 more weeks with Hoagland solution made without or with EDTA. Other treatments, lack of iron, or extra aluminium are given in Table S1. For rosette stage plants about 20 rosettes were used for one sample. Plant tissue was powdered in liquid nitrogen using a mortar and pestle, dried for 48 h at 60 °C, then kept 3 days in a desiccator before analysed by ICP (Inductive coupled plasma mass spectrometry) at NIBIO (Norsk institutt for bioøkonomi, Kjemisk laboratorier), Ås, Norway.

2.4. Total RNA extraction and RNAseq

After sowing on ½ MS medium with 1% sucrose without EDTA, the Petri dishes were placed at 4 °C for three days, followed by 5 days at 22 °C in 16 h light/8 h dark cycles. At this stage WT and *lcmt1* seedlings still looked similar and were considered suitable for harvesting 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 RNAseq (Illumina HISEQ 2500), three samples for both *lcmt1* and WT. Expression of the selected genes *PP2AA4*, *VBC35*, *IRT1*, *HSP90-1* were validated with qPCR. RNAseq expression analysis was performed using Bowtie transcriptome alignments, TopHat and Cufflink. FPKM (fragment per kilobase per million mapped reads), and fold change with p-values are listed for significant different expression values in *lcmt1* and WT ($p < 0.05$). Data are presented in Supplementary Table S2, FPKM is given in columns J and K, and fold change is given in column Q. Raw data sequences were deposited in SRA at NCBI, BioProject ID: PRJNA855460.

3. Results

3.1. Excluding EDTA from the growth medium leads to severe visual effects on lcmt1

To facilitate nutrient uptake, the chelator EDTA is generally added to the Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and Hoagland solution (Hoagland and Arnon, 1950). In early experiments we observed that lack of a chelator in the nutrient medium hampered growth of *lcmt1* more than was observed for WT (Fig. 1). This led us to look into effects of variable concentrations of minerals in the nutrient medium (Figs. 1–2) as well as their accumulation in the tissue (Table 1). After germination 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. 1a and b). To explore effects of stressful conditions on a later developmental stage, plants were cultivated in soil for three weeks, then moved to rock wool watered 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 on Hoagland solution 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), the stress symptoms were again more severe for *lcmt1*.

3.2. Non-optimal concentrations of micronutrients hamper root growth in lcmt1 stronger than in WT

The micronutrients manganese, iron and zinc act as cofactors in a wide range of enzymes and structures (Nishito et al., 1999). Such micronutrients may need to be complexed by compounds made by the plant itself or present in the root zone to enhance uptake, ensure distribution to different organs, and avoid harmful reactions inside cells. Since lack of the chelator EDTA had a high negative impact on *lcmt1* growth, we investigated if stress caused by non-optimal levels of iron and zinc would be aggravated in the *lcmt1* mutant. The concentration of iron ($FeNa_2EDTA$) was varied from 0 to 500 μM in the agar medium (Fig. 2a and b, Supplemental Fig. S1). Relative to control conditions (25 μM Fe-EDTA), shoot weight of seedlings was lowered by approximately 45 and 70% when exposed to 350 and 500 μM $FeNa_2EDTA$, respectively, but no significant difference in weight decreases was seen for *lcmt1* as compared with WT. However, for roots, at 200 μM and higher there was

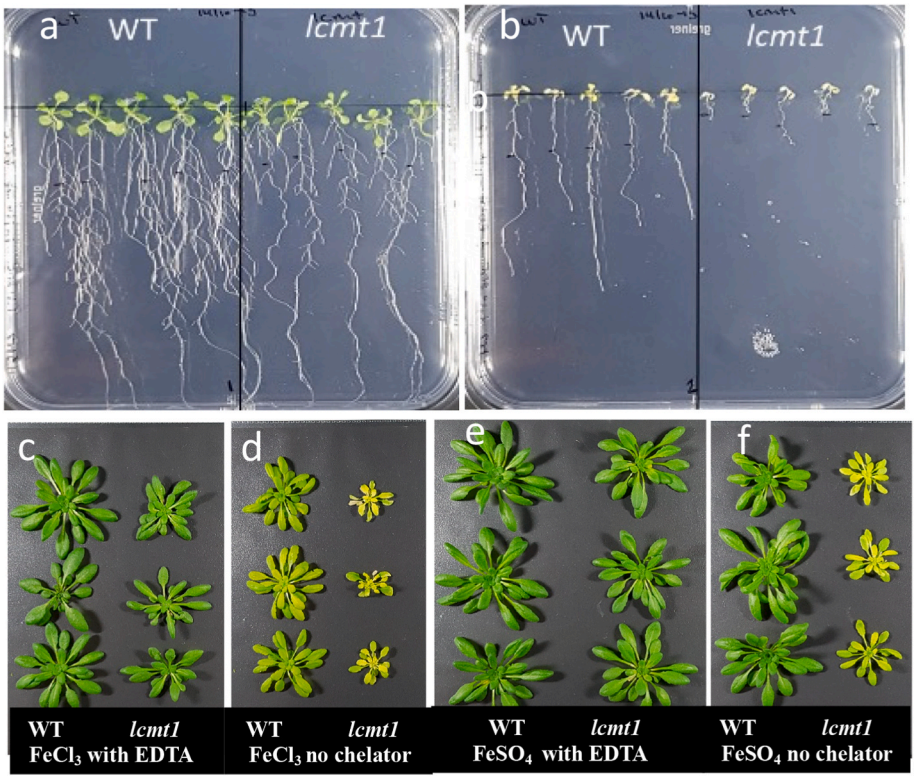

Fig. 1. WT and *lcmt1* grown on agar medium or rock wool with or without a chelator. After germination on complete MS medium, seedlings were grown for 7 days on **a)** control MS; **b)** MS without the EDTA chelator. After growth in soil for three weeks, plants were transferred to rock wool and grown for another three weeks with Hoagland solution modified to contain **c)** $FeCl_3$, with EDTA; **d)** $FeCl_3$, no chelator; **e)** $FeSO_4$, with EDTA; **f)** $FeSO_4$, no chelator.

a stronger negative effect on root elongation in *lcmt1* as compared with WT. At 500 μM, roots of both WT and *lcmt1* stopped growing (Supplemental Fig. S1). The results showed that high concentrations of $FeNa_2EDTA$ impaired root growth in *lcmt1* more strongly than in WT (Fig. 2b).

The effects of excluding EDTA from the agar medium in the presence or absence of 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. 2c and d). Addition of 16 μM zinc (standard concentration) led to increased fresh weight, but only when comparing media containing EDTA. A ten times higher concentration of zinc (160 μM) did not have positive effects. In conclusion, when avoiding micronutrients, shoot fresh weight of both WT and *lcmt1* was reduced, but there were no obvious differences between the responses of *lcmt1* and WT (Fig. 2c). On the other hand, root growth was stronger impaired in *lcmt1* compared with WT in all media lacking micronutrients and/or EDTA (Fig. 2d).

3.3. Aluminium in the growth medium affects lcmt1 more negatively than WT

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 et al., 2010). Shoot fresh weight of seedlings grown on agar decreased when $Al_2(SO_4)_3$ was added in concentrations of 0.4 or 0.6 mM, but with no significant difference in responses between WT and *lcmt1* (Fig. 2e). Roots of *lcmt1* grew significantly less when aluminium was present in comparison with WT (Fig. 2f, Fig. S2.).

3.4. Heat and oxidative stress influence lcmt1 more than WT

Temperature fluctuates throughout the day and night, and the global trend of increasing temperature will no doubt influence plant growth. Temperature strongly affects catalytic reactions, including enzymatic reactions, and high or low temperatures may disrupt the balance in metabolism. During the day, in strong sunlight, 37 °C is a temperature reached many places in the environment and is the heat treatment used here. After germination for 5 days, seedlings were exposed to 37 °C for 0–48 h, placed back at 22 °C, and assessed six days later. A heat treatment for 18 h or longer reduced seedling shoot fresh weight, but there was no significant difference between WT and *lcmt1* (Fig. 2g, Fig. S3). Negative effects on root growth were observed after 12 h of heat treatment (Fig. 2h), and after 24, 36 and 48 h the effects on root growth were stronger for *lcmt1* than for WT (Fig. 2h, Fig. S3). The influence of heat was also tested with plants grown in soil for 5 weeks before exposure to 37 °C for 0, 8, 18, or 24 h, and replacement to 22 °C (Fig. 3, Fig. S4). Pictures were taken immediately after heat treatment, after one week, and after two weeks. Both WT and *lcmt1* grew well after 8 or 18 h heat treatment (Fig. S4). After 24 h heat treatment and one week of recovery, WT showed more green leaves than *lcmt1* in three repeats (Fig. 3, Fig. S4). After two weeks, however, all plants died indicating that the shoot meristem had been impaired in both *lcmt1* and WT.

Reactive oxygen species (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).

3.5. Element analysis reveals perturbed sodium and boron levels in lcmt1, and high zinc and manganese levels in response to omission of chelator for both lcmt1 and WT

Lcmt1 and WT seedlings were grown in Petri dishes on agar with or without EDTA in the growth medium, and seedling shoots were harvested for element analysis. Element concentrations are given relative to dry weight of plant tissue (Table 1, Table S1). The ratios of

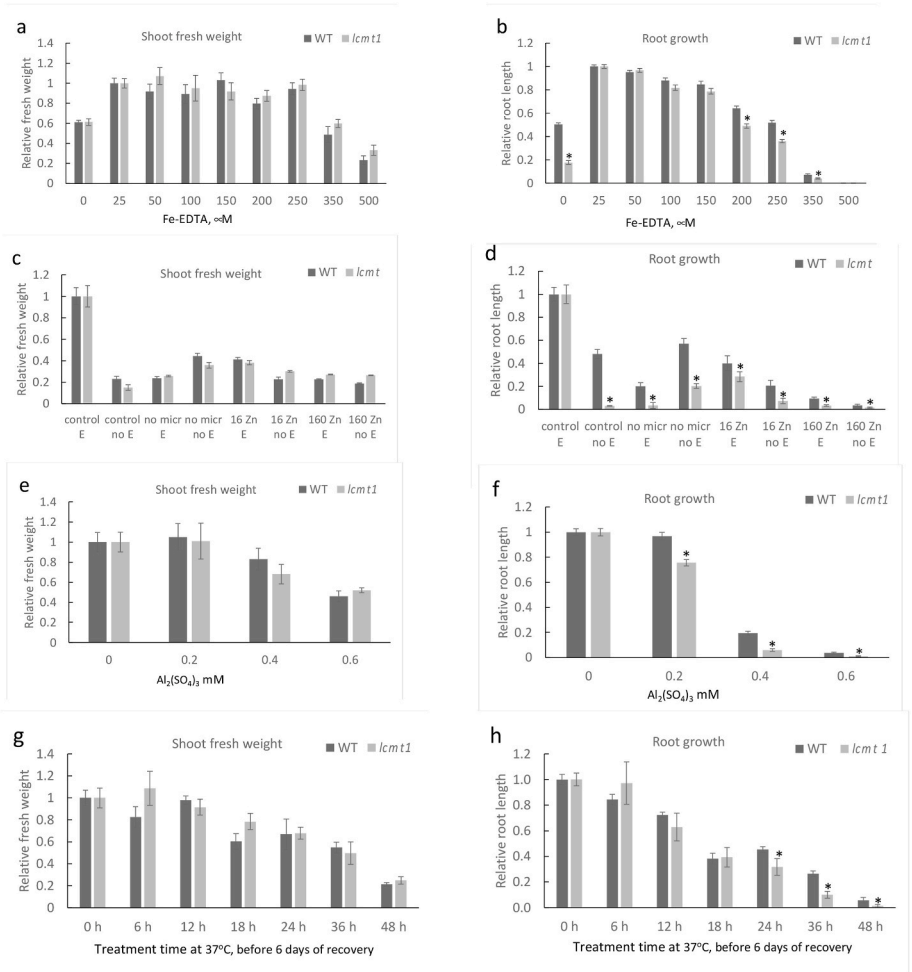

Fig. 2. Comparison of WT and *lcmt1* seedlings on MS agar medium modified with different concentrations of micronutrients, EDTA, aluminium, or heat treatment. **a, b)** MS media modified with different concentrations of Fe-EDTA ($FeNa_2EDTA$). **c, d)** MS media modified for micronutrients and EDTA: standard with EDTA (control, E); standard but no EDTA (control, no E); no micronutrient with EDTA (no micr, E); no micronutrient without EDTA (no micr, no E); 16 μM Zn but no other micronutrients, with EDTA (16 Zn, E); 16 μM Zn but no other micronutrients, no EDTA (16 Zn, no E); 160 μM Zn but no other micronutrients, with EDTA (160 Zn, E); 160 μM Zn but no other micronutrients, no EDTA (160 Zn, no E). **e, f)** MS medium modified with different concentrations of aluminium. **g, h)** Heat treatment at 37 °C for various hours and assessed six days thereafter. To be able to compare changes in growth due to different treatments, all data were normalized to the standard treatment (set to one) for both WT and *lcmt1*, i.e., normalized to the standard MS medium (a–f), and 0h timepoint (g–h). Mean values for standard treatment were 8.3 mg and 5.8 mg fresh weight per seedling for WT and *lcmt1*, respectively, and 75 mm root length for both WT and *lcmt1*. Data are averages of 30 seedlings (a, b) or 45 seedlings (c–g) for each treatment and genotype, SE is given, a star indicates significant differences between changes in WT and *lcmt1* by TTEST, for $p < 0.05$.

concentrations, i.e. element concentration in *lcmt1* divided by element concentration in WT, were calculated and are also listed in Table 1. Element concentrations were very similar in *lcmt1* and WT. With EDTA in the medium the measurements did not underscore any clear differences between *lcmt1* and WT, generally differences did not exceed circa 10%. When the agar medium had no chelator, differences were found for copper (up by 35%) and zinc (up by 20%) in *lcmt1* as compared with WT. To check for general effects of omitting EDTA, data for both *lcmt1* and WT grown with no chelator were pooled and compared with data for *lcmt1* and WT grown with EDTA. This pointed to a large and significant increase (48%) in zinc content in response to lack of EDTA in the growth medium (Table 1).

For bigger plants, i.e. rosettes of plants grown on rock wool, there were no significant differences in element levels between *lcmt1* and WT when grown in the presence of EDTA, with the exception of sodium which was significantly enhanced in *lcmt1*, by 44%. An enhanced level of sodium in *lcmt1* was persistent throughout all experiments with rosette leaves, also in experiments where iron was omitted, plants were grown in soil, or grown with excess aluminium (Table S1). The reason that agar-grown seedlings did not show such a strong increase in sodium content for *lcmt1* is likely due to the agar which is high in sodium and the young age of the plants. Interestingly, for rosettes grown in the absence of EDTA, the content of boron was lowered by 49% in *lcmt1* in comparison with WT. When WT was compared with itself ± EDTA, and *lcmt1* compared with itself ± EDTA, significant increases in the concentrations of Cu, Mn and S were observed for both genotypes in response to lack of EDTA.

3.6. Global transcript analysis shows up-regulation of metal homeostasis genes and down-regulation of defence genes in lcmt1

WT and *lcmt1* seedlings were germinated and grown without EDTA in Petri dishes and harvested at an early stage while the genotypes still looked very similar and considered suitable for comparison of changes in mRNA transcript levels. Almost 3 000 genes showed significantly different expression levels, 1528 genes were up-regulated and 1366 genes were down-regulated in *lcmt1* in comparison with WT. When the limit was set to two-fold different transcript levels, 520 genes were up-regulated and 653 genes were down-regulated in *lcmt1*. Several genes (155) were more than five-fold downregulated in *lcmt1* (Table 2, Supplemental Table S2).

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 2-fold 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–S5). Genes up-regulated in *lcmt1* were enriched in iron homeostasis genes, cutin biosynthesis processes and various stress-annotated genes. We had previously found by reverse transcriptase real time PCR that the transcript level for the iron and metal transporter/sensor IRT1 was elevated in *lcmt1*, and central iron homeostasis genes were further inspected in the RNAseq data (Fig. S6). These genes included five *BHLH* transcription factors and the *BRUTUS E3* ligase involved in regulation of iron homeostasis, two genes directly involved in uptake of iron and other metals

Table 1
The element content in shoots of *lcmt1* and WT, genotype and EDTA effects. Shoots of seedlings grown on half MS medium and shoots (rosettes) from older plants grown on rock wool with Hoagland solution were analysed. Both growth states were tested with and without EDTA. Element concentrations are given as μg per g dry weight plant tissue. For seedling shoots, there were two samples per genotype and treatment (each sample had 300 seedlings). The influence of omitting EDTA was analysed by comparing all samples from seedlings grown without EDTA (WT and *lcmt1*) to all samples with EDTA (WT and *lcmt1*). For rosette stage plants, four samples of each genotype and treatment were harvested (each sample had 20 plants). Standard error (±SE) is given (equals spread when only two samples). Ratios for element concentrations in *lcmt1* divided by element concentrations in WT are given. Ratios for element concentrations in plants without EDTA divided by plants with EDTA are also given. Ratios that were significantly different from 1 with $p < 0.05$ by TTEST are written in bold. (Raw data are given in Table S1).

Sample description	Measurement Calculations	B	Cu	Fe	Mn	Mo	Na	Zn	P	S
WT seedlings with EDTA, n = 2	Element μg/g	51.7 ± 1.3	2.8 ± 0.4	124.0 ± 7.6	300 ± 3.0	6.2 ± 0.1	8842 ± 258	126.4 ± 0.4	10340 ± 10	6605 ± 55
Lcmt seedlings with EDTA, n = 2	Element μg/g	56.9 ± 2.4	3.1 ± 0.6	119.3 ± 1.0	311 ± 11	5.8 ± 0.2	9969 ± 457	121.95 ± 1.75	9780 ± 14	7120 ± 100
	Ratio *lcmt*/WT	1.10	1.11	0.96	1.04	0.93	1.13	0.97	0.95	1.08
WT seedlings without EDTA, n = 2	Element μg/g	52.4 ± 0.7	3.1 ± 1.1	103.5 ± 6.6	271.5 ± 18.5	6.2 ± 0	9053 ± 320	166.9 ± 17.6	11415 ± 195	7450 ± 200
Lcmt seedlings without EDTA, n = 2	Element μg/g	53.4 ± 3.8	4.2 ± 1.8	109.5 ± 3.0	296.5 ± 24.5	5.9 ± 0.2	9431 ± 470	199.7 ± 25.3	10060 ± 180	7890 ± 80
	Ratio *lcmt*/WT	1.02	1.35	1.06	1.09	0.95	1.04	1.20	0.88	1.06
All seedlings, with EDTA, n = 4	Element μg/g	54.3 ± 1.9	2.9 ± 0.3	121.6 ± 3.4	305.5 ± 5.6	6.0 ± 0.1	9406 ± 389	124.2 ± 1.5	10060 ± 171	6863 ± 156
All seedlings, without EDTA, n = 4	Element μg/g	52.9 ± 1.6	3.7 ± 0.9	106.5 ± 3.4	284 ± 14.5	6.1 ± 0.1	9242 ± 256	183.3 ± 15.7	10737 ± 406	7670 ± 154
	Ratio −/+ EDTA	0.97	1.26	**0.88**	0.93	1.01	0.98	**1.48**	1.07	**1.12**
	P value	0.5753	0.4642	**0.0200**	0.2153	0.7080	0.7371	**0.0096**	0.1234	**0.004**
WT rosettes with EDTA, n = 4	Element μg/g	104.5 ± 8.7	9.3 ± 1.5	104.3 ± 16.4	80 ± 37	4.9 ± 1.1	273.8 ± 20.5	30.55 ± 7.4	4235 ± 1240	7115 ± 857
Lcmt rosettes with EDTA, n = 4	Element μg/g	105.9 ± 12.2	9.7 ± 1.6	164.3 ± 76.1	67.5 ± 31.5	4.8 ± 1.0.8	393.7 ± 34.5	31.6 ± 6.7	4080 ± 1019	8557 ± 641
	Ratio *lcmt*/WT	1.01	1.05	1.57	0.84	0.98	**1.44**	1.03	0.96	1.20
	P value	0.9211	0.8346	0.4098	0.7782	0.9376	**0.0113**	0.9082	0.9262	0.2264
WT rosettes without EDTA, n = 4	Element μg/g	120.1 ± 14.0	41.5 ± 9.3	114.9 ± 16.0	192.5 ± 47.6	9.9 ± 1.7	193.2 ± 32.5	53.2 ± 16.0	7085 ± 788	14520 ± 2194
Lcmt rosettes without EDTA, n = 4	Element μg/g	61.1 ± 6.8	39.4 ± 3.1	162.2 ± 28.4	177.8 ± 24.1	6.8 ± 0.4	373.5 ± 46.5	64.3 ± 13.2	9948 ± 302	16445 ± 614
	Ratio *lcmt*/WT	**0.51**	0.95	1.41	0.92	0.69	**1.93**	1.21	**1.40**	1.13
	P value	**0.0091**	0.8380	0.2418	0.7199	0.1286	**0.0284**	0.6319	**0.0146**	0.4283
WT rosettes, n = 4	Ratio −/+ EDTA	1.15	**4.48**	1.10	**2.41**	**2.03**	**0.71**	1.74	1.67	**2.04**
	P value	0.3169	**0.0059**	0.6179	**0.0276**	**0.0268**	**0.0482**	0.1876	0.1005	**0.0200**
Lcmt1 rosettes, n = 4	Ratio −/+ EDTA	**0.58**	**4.07**	0.99	**2.63**	1.42	0.95	2.03	**2.44**	**1.92**
	P value	**0.0186**	**0.0003**	0.9813	**0.0395**	0.0737	0.7622	0.0975	**0.0014**	**0.0001**

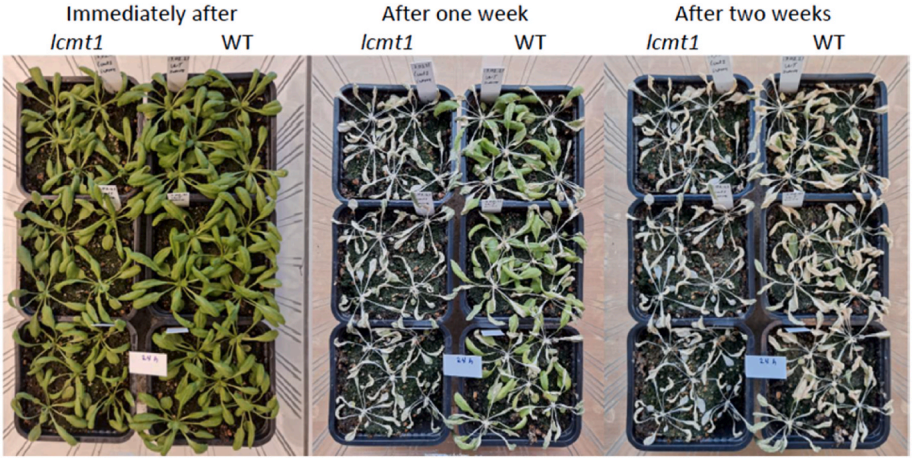

Fig. 3. Effects of heat treatment on WT and *lcmt1* rosette plants. Plants were grown in soil for 5 weeks, then treated with heat (37 °C) for 24 h, and thereafter placed back at 22 °C. Pictures were taken immediately after treatment, after 1 week, and 2 weeks recovery at 22 °C (more repeats are given in Fig. S4).

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 OPT3. *IMA* genes (*IRON-MAN 1, 2, 3, 4, and 6*) were up-regulated 4–5 times (not all included in Fig. S6). *IMA* genes were recently shown to be crucial for regulation of iron transport and homeostasis (Grillet et al., 2018). Three *NAS* genes (1, 2, 4) encoding enzymes that synthesise the chelator nicotianamine were upregulated, whereas the iron storage protein gene *FER1* was down-regulated in *lcmt1*, indicating less storage capacity. *ZINC-INDUCED FACILITATOR (ZIF1)* showed 2.9 times higher transcripts levels in *lcmt1*

Table 2
Number of genes differentially expressed in *lcmt1* and WT. Seedlings were grown in a 16 h light/8 h darkness regimen on half MS medium lacking EDTA.

	Number of genes
Genes significant differently expressed	2894
Genes upregulated in *lcmt1*	1528
Genes downregulated in *lcmt1*	1366
Genes > 5-fold different in *lcmt1* and WT	194
Genes > 5-fold upregulated in *lcmt1*	39
Genes > 5-fold downregulated in *lcmt1*	155
Genes > 2-fold different in *lcmt1* and WT	1173
Genes > 2-fold upregulated in *lcmt1*	520
Genes > 2-fold down-regulated in *lcmt1*	653

Table 3
GO (Gene Ontology) enrichment. Statistical over representation (powered by Panther, https://www.arabidopsis.org/tools/go_term_enrichment.jsp). Genes expressed at a level at least twice higher or lower in *lcmt1* as compared with WT were included in the analysis (Details are shown in Tables S3–S8).

	Number of genes	fold enriched
Biological processes 2-fold higher in *lcmt1*		
Iron homeostasis	10	13.88
Cutin biosynthesis process	6	18.97
Response to water deprivation	47	2.62
Response to osmotic stress	47	2.97
Response to stress	179	1.92
Molecular function 2-fold higher in *lcmt1*		none
Cellular component 2-fold higher in *lcmt1*		none
Biological processes 2-fold lower in *lcmt1*		
Photosynthesis, light harvesting in photosystem 1	10	19.02
Response to light stimulus	91	2.02
Defence response to other organisms	126	2.66
Response to stress	228	1.96
Response to ethylene	22	4.10
Response to salicylic acid	45	4.63
Response to oxygen levels	32	4.36
Response to fungus	67	3.00
Response to bacteria	70	3.02
Molecular function 2-fold lower in *lcmt1*		
Chlorophyll binding	9	14.75
Heme binding	29	3.56
oxidoreductase activity	62	1.96
Cellular component 2-fold lower in *lcmt1*		none

compared to WT (Table S2). ZIF1 is important for transportation of nicotianamine and zinc into the vacuole and critical for both zinc and iron homeostasis (Haydon et al., 2012). Many stress-related genes were differentially expressed in *lcmt1* and WT, they were either up (179) or down (126) -regulated. Genes involved in photosynthesis, especially light-harvesting, were strikingly downregulated in *lcmt1*. Also, genes encoding heme-binding proteins many of which are cytochromes, were down-regulated in *lcmt1*. Transcripts of the heat shock protein gene *HSP90-1* known to be involved in many different types of stresses was 2.2 times higher in *lcmt1* than WT (Table S2).

4. Discussion

The experiments defined several stress factors that reduced growth of *lcmt1* plants more strongly than WT plants. These factors were lack of chelator, lack of all micronutrients, or elevated concentrations of minerals in the nutrient medium (Fe, Zn or Al), heat exposure, and oxidative stress. When omitting a chelator, the *lcmt1* mutant showed hampered growth and chlorosis (Fig. 1), indicating impaired uptake or a disturbed balance of ions. Tissue element analysis showed a higher sodium content in *lcmt1* as compared with WT under various conditions (Table 1, Table S1). Salinity is a widespread and major stress that strongly reduces growth both in agricultural and wild plants (Gupta et al., 2021). Sequestration of sodium in the vacuole and excretion of sodium to the environment can help plants to tolerate salinity stress. The *LCMT1* gene was previously shown to be of importance for coping with salt stress because the *lcmt1* knockout mutant (also called *sbi1-2*) was more susceptible to 100 mM NaCl than WT, and knockout of the *LCMT1* gene was also correlated with demethylated PP2A in the work by Chen and co-workers (Chen et al., 2014). In the present work, element analysis of rosette stage plants showed that sodium content was strongly elevated in *lcmt1* compared with WT and strengthen the view that *LCMT1* plays a role in salt tolerance. The Na^+/H^+ EXCHANGER 1 (NHX1) is a main contributor to transport of sodium and potassium into vacuoles in Arabidopsis and essential for ion balance (Bassil et al., 2019). Interestingly, *NHX1* transcripts were significantly upregulated (by factor 1.7) in *lcmt1* relative to WT (Table S2). At the protein level, NHX1 and its homologues are known to be regulated by phosphorylation status in both plants and mammals (Hima Kumari et al., 2018), but phosphatases involved still need to be identified. The accumulation of sodium in *lcmt1* may also be linked with other transporters, and various membrane transporters are known to be regulated by phosphorylation/dephosphorylation and involved in salt tolerance (Gupta et al., 2021). Altogether, the involvement of *LCMT1* in resistance to NaCl treatment (Chen et al., 2014), effects on sodium content and *NHX1* transcripts in the *lcmt1* knockout (this work), point to salinity stress as a factor that can have contributed to the conservation of *LCMT1* in plants.

The element analysis showed that boron concentration in rosette stage *lcmt1* was halved in comparison with WT, under stressful conditions caused by lack of EDTA. Boron is a constituent of cell walls where it covalently links pectin chains together. Additionally, boron has other functions, including some regulatory functions, in both plants and animal. Boron is important for pollen development, and in animal embryos development stopped by knockout of a boron transporter, a homolog of the Arabidopsis BOR1 (Goldbach and Wimmer, 2007; Park et al., 2004). Under optimal growth conditions, passive diffusion is sufficient for acquiring boron in plants, but boron deficiency as well as toxicity is a common problem in agriculture in very wet or arid areas, respectively. Boron homeostasis in plants is achieved by boric acid channels (NIPs) and borate exporters (BORs), both localized in a polar way in cells. Arabidopsis NIP5; 1 is on the soil side of the plasma membrane, whereas BOR1 is localized towards the steel (Matthes et al., 2020; Yoshinari and Takano, 2017). The decreased level of boron in *lcmt1* rosettes stage plants and changes in expression of *BOR2* and *BOR4* (by factors 1.5 and 3.0, respectively, Table S2) point to a role for *LCMT1* in boron homeostasis.

In rosette stage plants under standard conditions, copper, manganese, and zinc summed up to a concentration lower than iron (lower by 17%). In contrast, in plants grown without EDTA these elements summed up to a concentration twice that of iron (based on data from Table S1). Evidently, WT coped better with the high levels of Cu/Mn/Zn than *lcmt1*. In mammals, excessive exposure to manganese was associated with oxidative stress and autophagy dysfunction, and the manganese effects were linked with downregulation of PP2Ac methylation and the conserved mTORC1 pathway (Xu et al., 2021). Interestingly, such signalling pathways are also conserved in plants (Reumann et al., 2010), and our results give a starting point for investigating connections of manganese, PP2Ac methylation and the mTORC1 pathway also in plants.

The *lcmt1* plants showed the visual signs and gene activations compatible with iron deficiency (Table 1), although element analysis did not indicate any lack of iron. Genes typically induced by iron deficiency (Fig. S6), included three of the four Arabidopsis *NAS* genes. *NAS* genes

encode for the enzyme nicotianamine synthase which catalyses the formation of nicotianamine from three molecules of SAM. Nicotianamine is found in all plants, and makes complexes with Fe, Zn, Mn, and Cu. Nicotianamine is essential for internal transport of such elements, for distribution to metal-dependent proteins and structures, as well as for detoxification and accumulation of excessive elements in the vacuole (Seregin and Kozhevnikova, 2020). In Arabidopsis, quadruple knockout of *NAS* genes was lethal (Schuler et al., 2012). Various types of iron homeostasis genes were induced in *lcmt1*. The iron/metal sensor and transporter IRT1 binds not only iron, but other metals, like Zn and Mn which lead to phosphorylation, ubiquitination, internalization and finely degradation of IRT1 (Dubeaux et al., 2018; Martín-Barranco et al., 2020). Furthermore, BTS and IMA proteins bind also Zn (Kobayashi, 2019). Such non-iron metals reached much higher levels in plants grown in the absence of a chelator (Table 1), hence are important for the induction of iron/metal homeostasis genes. Why the induction of such genes is much stronger in the absence of *LCMT1* still needs to be sought.

PP2A has generally been shown to be a negative regulator of defence in Arabidopsis and other plants (Durian et al., 2016; Kataya et al., 2015; Segonzac et al., 2014). Although PP2A activity is slightly decreased (by about 20%) in the *lcmt1* mutant (Creighton et al., 2017), a defence response was not triggered in the *lcmt1* mutant. On the contrary, *LCMT1* acts rather as a positive regulator of defence since defence genes were down-regulated in the mutant (absence of *LCMT1*) (Table 3). Although not much studied in plants, methylation of PP2A catalytic subunits have been found to favour binding of specific regulatory B subunits (Janssens et al., 2008). According to Janssen and collaborators methylation favours binding of the type of subunits called B55, while B′ binds equally well to non-methylated PP2Ac. In *lcmt1* which has only, or mainly, non-methylated PP2Ac this may promote binding of B′ subunits in the PP2A holoenzyme. The B′ subunits are the type of subunits identified as participating in the PP2A holoenzyme that keeps the BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE) pathogen receptor inactive by dephosphorylation, thereby avoiding unnecessary activation of defence genes in the absence of pathogens (Segonzac et al., 2014). Furthermore, a B' subunit was also recognized as important for avoiding unnecessary immune responses in leaves of Arabidopsis (Trotta et al., 2011). Hence, the predominant presence of non-methylated PP2Ac in *lcmt1* may shift the balance of regulatory B subunits in the PP2A holoenzyme towards those important for harnessing defence genes.

In conclusion we have identified various stressful conditions with negative effects on growth that were aggravated by the loss of *LCMT1*, for instance lack of a chelator in the growth medium. The phenotyping of *lcmt1* and WT, together with element analysis of plant tissues and RNAseq data substantiate a role of LCMT1 in metal homeostasis and defence responses to other organisms, and points to genes affected.

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. All authors have read and approved the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw data sequences were deposited in SRA at NCBI, BioProject ID: PRJNA855460.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jplph.2022.153843.

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