University of Stavanger

Faculty of Science and Technology

BACHELOR`S THESIS

Study program/Specialization: Bachelor degree in Biological Chemistry	Spring semester, 2021 Open / Restricted access
Writer: Stine Åreskjold Nordbø & Thea Kleiberg	Thea Kleiberg Stine Å Nordbø (Writer's signature)
Faculty supervisor: Cathrine Lillo, Dugassa Nemie-Feyissa External supervisor(s):	
Thesis title: Methylation of PP2A in *Arabidopsis thaliana* - effects on gene expression and phenotype	
Credits (ECTS): 20	
Key words: PP2A, LCMT1, Arabidopsis thaliana, methylation of PP2A-C, nutrient deficiency, oxidative stress and gene expression analysis	Pages: 52 + enclosure: 15 Stavanger, 20/06/2021 Date/year

Acknowledgements

We would like to thank our supervisor Professor Cathrine Lillo for the great support and opportunity to work on this interesting project. We are grateful for all the help and advice professor Cathrine has given us both in writing and in the laboratory. We would also thank Ph.D. Dugassa Nemie-Feyissa for the continuous help, great knowledge, and patience in the lab. We would also like to thank MA student Nabeela Zaman for giving us good advice and showing us different techniques in the lab, and other students working at the lab for being so helpful and welcoming. Finally, we would like to thank our family and friends for all the support and encouragement throughout this period.

Abstract

Methylation of the carboxyl group in PP2A and formation of the PP2A holoenzyme is a highly regulated process. The catalyzer of this process is Leucine carboxyl methyl transferase 1 (LCMT1). PP2A-C methylation is essential for the health of eukaryotic cells. In the knockdown *lcmt1*, only the unmethylated form of PP2A-C is present. It is expected for this knock-out *lcmt1* to react poorly under stress conditions in comparison to WT which still holds the LCMT1 gene. The mutant *lcmt1* grew in the same manner as WT in germination media. Further, we investigated how WT and *lcmt1* reacted to nutrient deficiency and to the addition of H_2O_2, which induced oxidative stress. The importance of plants ability to adapt to the environment is important for the growth under conditions with deficiency of nutrients. The deficiency of nitrogen (N) and phosphorus (P) can have many negative consequences for crop production and can reduce yields. Visible traits of this deficiency are paler leaves from chlorosis or purple color from anthocyanin production. Potassium (K) has shown to be an important nutrient for the plant to grow under stressful conditions, where the tip of the leaves could be affected by chlorosis. This experiment showed that both WT and *lcmt1* were affected by a lack of nitrogen and phosphorus. The leaves turned out paler/yellow and some had a purple color that could indicate anthocyanin production and chlorosis. Without P, the roots were shorter and without N the plant weight was lower than in media containing these nutrients. The deficiency of K did not seem to have any effects on the plants in this study and needs to be more researched. The oxidative stress experiment can show how important the methylation of PP2A-C in *A. thaliana* is under stress conditions. The results from treatment with H_2O_2 fluctuated. WT weighed more and had more elongated shoots with the addition of H_2O_2 compared to *lcmt1*. The mutant growth was impaired and showed the most inhibition. We also examined the expression of the genes involved in ROS scavenging like *APXs* and *FSD1*. *HSP90* was investigated as it is involved in various stress responses. All genes were expressed at high levels in WT and *lcmt1* in both the control and in the stressed treatment. WT induced with 0.5 mM H_2O_2 had higher expression of *APXs*, *FSD1*, and *HSP90* in comparison to *lcmt1* with the same treatment. These differences in gene expression in stress environments may show how important the PP2A-C methylation is for cell survival and health in these situations.

Abbreviations

- ½ MS: half strength of regular Murashige and Skoog medium
- ACT8: ACTIN 8
- APXs: Ascorbate peroxidase chloroplastic stromal
- cDNA: complementary DNA
- Ct: Cycle threshold
- Cq: Quantification cycle
- EDTA: Ethylenediaminetetraacetic Acid
- FSD1: Fe-Superoxide Dismutase 1
- GuHCl: guanidine hydrochloride
- GTC: guanidine thiocyanate
- HSP90: Heat Shock Protein 90
- *lcmt1:* leucine carboxyl methyltransferase 1 knockout mutant
- PP2A: Protein phosphatase 2A
- RNA: Ribonucleic Acid
- ROS: Reactive oxygen species
- RQ: Relative quantification
- RT-PCR: Real Time Polymerase Chain Reaction
- SD: standard deviation
- SE: Standard Error
- UBC35: Ubiquitin- Conjugating Enzyme 35
- UBQ: ubiquitin
- WT: wild type

Table of contents

1. Introduction

1.1 Arabidopsis thaliana

For the last thirty-seven years, *Arabidopsis thaliana* has been the first choice of model plant in plant biology. The diploid plant *Arabidopsis thaliana* has self-fertilizing and have bisexual flowers. *A. thaliana* was the first plant to have its genome fully sequenced. *A. thaliana* is able to give an efficient genetic analysis in short time, and because of its small size it can grow fast in small space in a laboratory such as a Petri dish (Koornneef *et al*.,2010).

1.2 Protein phosphatase 2a

The responses of several internal and external signals in *A. thaliana* are handled by Protein phosphatase 2a (PP2A) among others. The complex protein phosphatase type 2A has three distinctive subunits. The scaffolding (A), the regulatory (B), and the catalytic (C) subunit. The catalytic core consists of the catalytic and scaffolding subunit, the dephosphorylation occurs here. The regulatory subunit controls the localization of PP2A in the cell and is particular to the substrate. In plants the regulatory subunit is divided in to three different groups: The B/B55, the B′ and the B′′ (Creighton, *et al*., 2017; Lillo, *et al*., 2014).

A. thaliana has 5 C, 3 A, and 17 B subunits in the PP2A complex. This provides 255 combinations in the complex. The regulatory subunit as mentioned earlier is divided into three groups, in the *A. thaliana* the B/B55 has two, B′ has nine, and B′′ has six members. Reversible methylation of the C subunit is one of the processes that control PP2A activity (Creighton, *et al*., 2017).

PP2A dephosphorylates protein, and thus controls the activity of many proteins. PP2A is an enzyme that is important in several processes in cell signalling and is linked to responses to stress. The enzyme is formed from different subunits. The subunit can form a core dimer of subunit A and C, or a trimer when B also is connected and accordingly then create the PP2A holoenzyme. PP2A can control the activity of the protein, because of these subunits. The number of subunits differs in different species. The regulation of PP2A is controlled from the formation of the holoenzyme, and covalent modifications after translation, and interaction between proteins. Furthermore, inhibitors, modulators, and activators also are factors that

PP2A relies on. Inhibitors that are specific for PP2A are Inhibitor-1^{PP2A} and Inhibitor-2^{PP2A}, both heat stable and the only inhibitors that inhibit PP2A (Creighton *et al*., 2017).

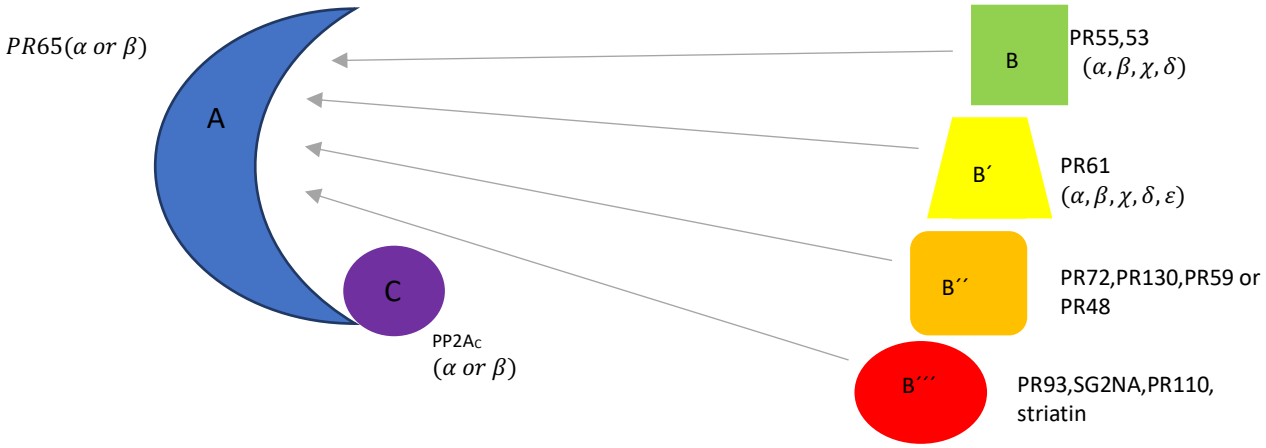

Figure 1.2.1: Configuration of the PP2A in *A. thaliana*. The scaffolding subunit (A), regulatory subunit (B), and catalytic subunit (C) are shown here. The A and C subunits make up the AC dimer, when also connected to subunit B the PP2A holoenzyme is created. Subunit A are encoded by PP465 and two related genes (α *or* β). The catalytic subunit is encoded by $PP2A_C$ and two related genes (α *or* β). The regulatory subunit (B) is encoded by two related genes (α *or* β) and encodes also PR55 and PR53. B´ is encoded by PR61 and five related genes. The B´´ is encoded by four genes PR72, PR130, PR59, or PR48. B´´´ is encoded by PR93, SG2NA, PR110, and striatin. The figure of PP2A structure is based on Sultana *et al*., 2016; Creighton *et al*., 2017; Lillo *et al*., 2014

1.3 LCMT1 Arabidopsis thaliana

Crucial for the health of plant cells is the appropriate configuration of PP2A holoenzymes. The methylation of the carboxyl group on the PP2A catalytic subunit is significant for the regulation of the gathering of the holoenzyme. The catalysator of this methylation is Leucine Carboxyl Methyl Transferase 1 (LCMT1). LCMT1 is an enzyme necessary for the survival of the cell (Stanevich *et al*.,2011). Lcmt1 is helped by SAM cofactor S-adenosyl-L-methionine, which is a methyl donor. Knockdown of LCMT1 from *A. thaliana* affects LCMT1´s ability to methylate the C-subunit (Creighton *et al.,* 2017). In the mutant *lcmt1*, only the unmethylated form of PP2A-C is present, and in mammalian cells induced cell death. This knock-out of LCMT1 has been linked to neurodegenerative diseases (Stanevich *et al*.,2011).

1.4 Anthocyanins

Anthocyanins are naturally in plants and classify as flavonoids that give colour to different flowers and fruits. They also provide photo-protection and antioxidant activity. It has been reported that anthocyanins are involved in osmoregulation. Additionally, they are great

regulators of responses to stress and the development of plants (Asad *et al.*, 2014). *Arabidopsis thaliana* can induce over 20 types of anthocyanins, it is proposed that the different types have distinctive roles (Kovinich *et al*., 2015).

Anthocyanins get induced by stresses from the environment, for example of drought or UV radiation or at low temperatures. Observations from Mita et al.,1997 of *Arabidopsis thaliana* that were exposed to osmotic stress, accumulated anthocyanins in larger amounts than the control plants (Asad *et al*., 2014).

1.5 Plant nutrition

The phenotype is visible traits, such as colour, growth, etc. In-plant nutrition phenotypes are observed to see how the plant reacts to deficiencies of different nutrients. The nutrients in plants consist of macro-and micronutrients. The macronutrients are most essential because the plant needs them in large amounts. Some of the important macronutrients that the plant needs are nitrogen (N), phosphorus (P), and potassium (K). The deficiency of different macronutrients can have many negative consequences for crop production, and this can lead to reduced yields and poor quality of food (Bang *et al*., 2020).

1.5.1 Limitation of nitrogen (N), potassium (K) and phosphorus (P)

Plants are often influenced by the season, where light, temperature, water, and nutrients greatly impact growth. Plants are often exposed to stress caused by the environment, and it is important to find out strategies to maintain growth under changing conditions. Nutritional stresses are one of the most limiting growth factors and are caused by a deficiency of nitrogen and other nutrients such as phosphorus and potassium (Massaro *et al*., 2019).

1.5.2 Limitation of Nitrogen

Nitrogen (N) is the most essential element after carbon (C), hydrogen (H), and oxygen (O). It is therefore the most essential mineral that has to be taken up by the soil. The root uptake of nitrate (NO_3) in higher plants such as *A. thaliana* is the main uptake of nitrogen. (Lea and Morot-Gaudry, 2001). Optimal plant growth occurs when both nitrate and ammonium (NO_3^- and NH_4^+) are present because then the plant can benefit from both of them. Both NO_3^- and NH_4^+ present in the plant gives better pH control. This is because the hydroxyl ions from NO_3^- uptake can compensate for the protons in NH_4^+ uptake. When plants are grown on a

medium containing both NH_4^+ and NO_3^-, it is possible to take up more nitrogen than one of them does alone (Vessey *et al*., 1990; Kronzucker *et al*., 1999; Lea, and Morot-Gaudry, 2001).

A visual symptom of nitrogen deficiency in *A. thaliana* is purple/reddish colour because of anthocyanin production. With the deficiency of nitrogen in *A. thaliana,* some key transcription factors are being expressed such as anthocyanin pigment 1 (PAP 1) and PAP 2. These transcription factors regulate anthocyanin synthesis (Xu *et al*., 2015; Bang *et al*., *2020).* PAP1 is known for the expression of genes in the flavonoid pathway. Genes in the anthocyanin pathway indicated to be an enhanced expression in an experiment done by Lillo, Lea, and Ruoff, 2007 including glycosyltransferases, acyltransferases, and glutathione S-transferases. Almost all of these genes were enhanced during nitrogen or phosphorus deficiency. The deficiency acted on the genes through the PAP transcription factors. A homolog to PAP1 is PAP2, and it can have the same effect on the flavonoid pathway as PAP1 (*Borevitz et al., 2000).* PAP2 is enhanced strongly by the depletion of nitrogen and phosphorus (Scheible *et al.,* 2004; Misson *et al.,* 2005; Lea *et al*., 2007; Mor- cuende *et al.* 2007; Lillo, Lea, and Ruoff, 2007).

The importance of PAP1 is in the sucrose-mediated activation of genes in the flavonoid pathway (Teng *et al.,* 2005; Diaz *et al.,* 2006; Solfanelli *et al.,* 2006; Lillo, Lea, and Ruoff, 2007). In the same experiment on rosette leaves of *A. thaliana*, PAP2 was highly induced under nitrogen deficiency, and also the leaves also had a higher amount of PAP2 transcripts than PAP1, this was shown by Real-time PCR (Scheible *et al.,* 2004; Lea *et al.,* 2007; Lillo, Lea, and Ruoff, 2007). Other symptoms can be reduced growth, leaf expansion can be inhibited, and reduced branching (Rayayu *et al*., 2005; Bang *et al.,* 2020). Another phenotype of limited nitrogen is chlorosis and it shows that the leaves turn out paled or yellowish (Forchhammer and Schwarz, 2018).

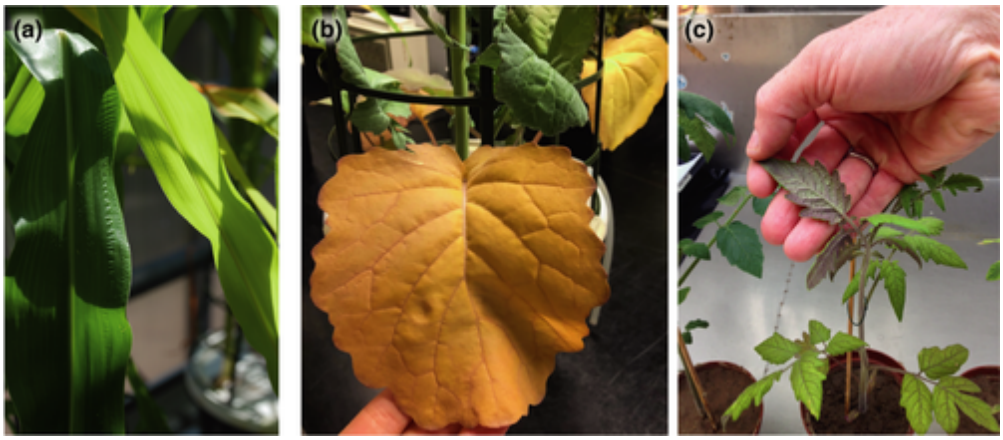

Figure 1.5.1: N deficiency in maize, oilseed rape leaf and in tomato that first is shown on old leaves. a) The maize plant leaf to the right is healthy, and the one to the left is with low N. b) Oilseed rape leaf that shows anthocyanin accumulation when there is low N and chlorosis. C) Tomato plant with anthocyanins in veins and on abaxial side (Bang *et al.,* 2020).

1.5.3 Limitation of phosphorus

Phosphorus (P) is important in energy metabolism (ATP, NADPH) in nucleic acids and phospholipids. Plants take up Phosphorus mostly as $H_2PO_4^-$ and HPO_4^- (inorganic orthophosphate ions). P is important in the electron transport chain in photosynthesis and it needs light to produce ATP and NADPH in the thylakoid membrane. It is also important in the Calvin-Benson cycle where ATP and NADPH use CO_2 to make carbohydrates. Deficiency in P can cause low chloroplast Pi levels because Pi is one important substrate for photosynthesis, and therefore ATP production reduces. A visual symptom of lack of P is also purple/reddish leaves because of anthocyanins (Bang *et al.,* 2020).

Figure 1.5.2 P deficiency of P in leaf on maize plant and in tomato leaf, anthocyanosis first shown in older leaves. a) Leaf of maize plant with low P showing anthocyanosis b) Anthocyanin showing on the abaxial side of a tomato leaf (Bang *et al.,* 2020).

1.5.4 Limitation of potassium

Potassium (K) is also an essential macronutrient and has an importance in plant growth, metabolic function, stress tolerance, and agriculture. Some researches show that K^+ uptake is needed for a plant to respond to stress and for systematic growth response in *A. thaliana*. K^+ is essential for the activity of many enzymes and helps with turgor regulation which is important for cell volume growth and plant movements. Early symptoms that are visual in the shoot are chloroses at the tip of old leaves which can turn out brown/yellow (marginal necrosis) (Bang *et al*., 2020).

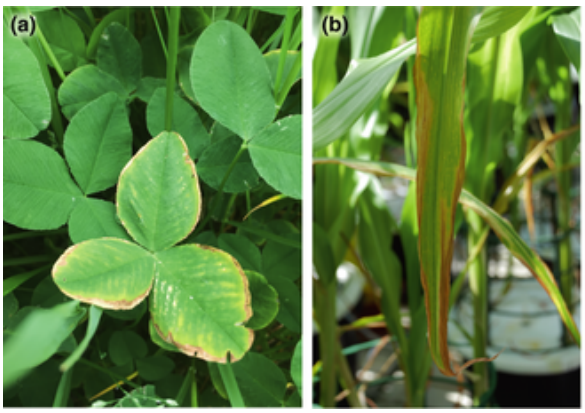

Figure 1.5.3 Potassium (K) deficiency results in marginal necrosis in a) white clover and b) maize (Bang *et al.,* 2020).

1.6 Adaptations that can reduce nutrient limitation

Plants have evolved different adaptations to cope with the deficiency of nutrients and increase uptake. One important adaptation is mutualism between plant roots and nitrogen-fixing bacteria. Others are mycorrhizal associations between plant roots and fungi that can supply phosphorus and other elements to plants (Campbell *et al.,*2018). Nitrogen deficiency can lead up to weakened development, leaf chlorosis, and worse quality in plants. Higher plants have developed mechanisms to adapt to environmental changes, but most mechanisms are still unknown (Massaro *et al*., 2019).

Under chlorosis, the cells are waiting for the nitrogen to be restored. The phenotype of chlorosis can be paled or yellowish leaves (Forchhammer and Schwarz, 2018). Chlorosis usually is discovered first in the oldest leaves and happens because the plant breaks down

chlorophyll and transports the nutrients to younger, growing tissues (Anon, 2020). In other words, the leaves use more nutrients of the short supply (Campbell *et al.,*2018*)*

In an experiment with nitrogen deficiency, nitrate reductase activity was lowered, but glutamine synthase activity increased because of a higher accumulation of cytosolic enzyme. Compared with increased nitrogen, there was a decrease in levels of proline, asparagine, and glutamine. This limited nitrogen can trigger high modifications of seed quality (Lemaitre, 2008). Low nitrogen status for over a long time can cause inhibition of lateral root growth and is controlled by nitrate transporter 1.1 (NRT1.1). NRT1.1 removes auxin from this lateral root for inhibiting (Krouk *et al*., 2010; Bouguyon *et al*., 2015; Bang *et al*.,2020). *NRT1.1* is a gene and is important in nitrate acquisition and signaling and can facilitate nitrate assimilation of many concentrations of nitrate (Liu *et al*., 1999). It has also shown that it can function as a nitrate sensor that can regulate gene expression of other transporters such as NRT2.1 (Krouk *et al.*, 2006; Ho *et al*., 2009) and helps with the nitrate-regulated auxin translocation. NRT 1.1 is important both in the high and low affinity of nitrate absorptions and by this, the plant can adjust the root architecture (Sun and Zheng, 2015).

An experiment compared four generations of N-limited *Arabidopsis thaliana* with N sufficient because one wanted to see if there was a transgenerational memory. The results showed in RNA_{seq} analyses were enduring modulation of genes in downstream generations. Some modulations of signaling and transcription factors may indicate that there was a complex network present that maintained the expression of genes that respond to the status of N. N-responsive genes could be *NRT2.1, NIA1, and NIR*. Signaling and transcription factors could be NIGTs, NFYA, and CIPK23. This indicated that the plants changed with different N availability. The fourth generation was exposed to N deficiency and showed stimulation of N limitation responses. This could suggest that there was a possible involvement of transgenerational memory and plants can adapt to different N concentrations and this can give better opportunities for the next generation that could grow in the same stress conditions (Massaro *et al*., 2019).

Phosphorus deficiency can lead to the plant responding by increasing the ratio of the root to the shoot and changing the architecture of the root to use the phosphorus resources better. This is happening the most in the topsoil where there often is less availability of phosphorus. *A. thaliana* responds to the deficiency by changing the growth of the primary root, increasing the root hair frequency and length of lateral roots. *A. thaliana* responds to the limitation of

phosphorus by root apical meristem (RAM) exhaustion that leads to a reduction in the growth of the primary root. It has been shown that Fe^{3+} in the root tip is important for this inhibition (Ward *et al.,* 2008). Fe triggers STOP-ALMT1 module where exudation of malate is happening in the apoplast in the tip of the root. Malate gives strong Fe complexes and gives toxicity in the RAM tissue and resulting in the production of reactive oxygen species (ROS). The collection of ROS can stimulate callose formations and has an impact on the cell walls, stiffening them and results in prevented cell elongation in RAM. Mutations in the STOP1-ALMT1 can result in the primary root restore of growth when there is not enough P (Belzaergue *et al*.,2017). Root hair formation and elongation are caused by elevated auxin levels in the trichoblast that can trigger gene expression and auxin is an important regulator of the architecture of the root. When there is a limiting P, the auxin receptor TIR1 is upregulated and increasing the sensitivity of pericycle cells. This results in activation of auxin response factor (ARF) and this response factor promotes lateral root emergence. The auxin accumulation in Ram tissue during low P triggers the formation of trichoblast cells to form root hairs when the levels of auxin are upregulated at the root tip (Bang *et al.,* (2020).

From a study by Sustr, Soukup, and Tylova., 2019, the deficiency in K^+ adjusts auxin transport and RAM maintenance pathways decrease the activity of the meristem. This could happen partially through ethylene and NO signaling. The regulation is complex and needs further clarification because current knowledge is still incomplete.

Nutrient stresses may also alter DNA methylation. In a study by Mager and Ludewig., 2018 with maize roots, it was compared a part of the DNA methylome of nitrogen- and phosphorus deficiency and was analyzed by gene expression. There was a huge loss of DNA methylation under the limitation of nitrogen, but much less than this in the phosphorus limitation plant. DNA methylation changes are associated with adaptation to limiting phosphorus in *A. thaliana* because it can adapt to this condition by regulating genome accessibility. Previous research showed that nitrogen and especially phosphorus limitation could affect the DNA methylation but results from *A. thaliana* did not reveal a common understanding of the underlying mechanism yet.

1.7 Oxidative stress

The production of reactive oxygen (ROS) is correlated to oxidative stress. ROS consist of components such as superoxide radical (O_2-), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), and lastly a hydroxyl radical (OH) (Wu and Wang, 2019). Reactive oxygen is formed when a single electron of oxygen is reduced, and thus superoxide, hydrogen peroxide, and hydroxyl radicals are generated. When the rate of ROS production is high, the cell defence systems such as antioxidant defences fail to detoxify them. ROS will then oxidize several key cellular macromolecules at an increasing rate. Target macromolecules stop functioning as normal, which leads to changes in cell homeostasis. Lipids and proteins can get oxidized and result in oxidative stress of the cell. ROS can also be produced through an alteration of the oxygen electron so-called spin states. The process of this production is by photoactivation which creates singlet oxygen (Kader *et al.,*2009).

ROS is a by-product of effective energy production in plants when molecular oxygen is used for this metabolic process. Accumulation of ROS is toxic for the cell and is particularly reactive. Therefore, a cell needs mechanisms against ROS concentrations. Cells have antioxidant systems that control the accumulation of ROS and its concentration. The antioxidant systems are important to maintain the cell's homeostasis. Plants are not able to move and therefore need mechanisms similar to this, when stresses are introduced from their environment, to survive and grow. A slow rate of growth is also a coping mechanism plant utilize for adapting to changes in their environment in the forms of stress (Kader *et al.,* 2009).

Hydrogen peroxide (H_2O_2) is an element of ROS that are made by plants, both in stress situations and during growth. H_2O_2 controls the growth of plants and their progress at a low level but can-o damage to cells in plants at high concentrations. The gene L-ascorbate peroxidase (APXs) has large correspondence with hydrogen peroxide and has a role as scavenging H_2O_2 (Wu and Wang, 2019).

1.7.1 The genes APX, FSD1 and HSP90-1 roles in ROS

The component of ROS, that is capable of crossing the membrane by the aquaporins in the plasma membrane is H_2O_2. For this reason, H_2O_2 can move from different sites with the water. Peroxisomes are the organelles with the highest rate of hydrogen peroxide production in the plant cells, chloroplast comes second, and then mitochondria. High levels of hydrogen peroxide induce oxidative stress and do damage to important biological macromolecules.

Antioxidant enzymes protect the cells from the damage of oxidative stress. The gene APXs encodes an antioxidant enzyme, which degrades H_2O_2. Anthocyanins essentially help APXs and other enzymes to scavenge hydrogen peroxide (Wu and Wang, 2019).

APXs activate the reaction of the conversion of H_2O_2 into H_2O and L-dehydroascorbate, this occurs with electron donor ASC in both the water to water cycle and the ASH-GSH (ascorbate-glutathione) cycle. A study from Wu and Wang, 2019 proposed that *APX1* had an important role in protecting of functions of the chloroplast in *A. thaliana.* When abiotic stress is induced like salinity, excess light, heat, etc. The *APXs* gene may be expressed at different levels (Wu and Wang, 2019). *Arabidopsis thaliana* has eight different types of *APX,* in this study we used chloroplastic stromal type *sAPX* same as *APXs.*

1.7.2 FSD1 response in oxidative stress environment

FSD1 is an enzyme that removes superoxide radicals and responds to oxidative stress, it is also a part of the superoxide dismutases called SODs. Three isoforms of FeSOD are encoded in *Arabidopsis thaliana* genome (Dvorak et al., 2020). The FSD1 enzyme plays a key role in detoxifying high concentrations of ROS in plant cells. Localization of FSD1 in Arabidopsis are in the rosette tissue and chloroplasts (Kliebenstein *et al*., 1998)

1.7.3 HS90-1 role under applied stress conditions in plant cells

In a study by Bharadwaj., et al 1999, the response from heat shock is followed by an amplified rate of synthesis of heat shock proteins, these inhibit denaturation of several cellular proteins under various stress circumstances. Situations of stress can come at any time and can be impulsive, therefore genes like HSP90-1 are essential. Heat-shock protein (HSP) has initially been seen as an enzyme related to heat stress response, however, it is actually linked to many types of stress responses in plant cells including oxidative stress. HSP90 interreacts with R proteins (resistance proteins) and controls their stability, this is crucial for signal transduction in terms of a functional defence. In mammal cells, HSP90 role is as a signalling component, and it has been proposed that this applies in plant cells too. HSP90-1 reacts with SGT1b and RAR1 and forms a complex. This is necessary for the regulation of the RPS2 (Jacob *et al.,* 2017).

1.8 Real-Time PCR (RT-PCR)

Polymerase Chain Reaction (PCR) is a technique that can determine the amount of a target sequence, nucleic acid, or gene in a sample (Scientific, 2015). Real-time RT-PCR is different from conventional RT-PCR because it measures the amplified PCR product at each cycle and reliable data is more provided. Real-Time PCR collects data in the exponential phase in Real-time and results in precise measurements of the starting template (Gachon *et al*.,2004). The Real-time PCR platform has a thermal cycler, optics for fluorescence excitation and one for collection of emission, and a computer and software for the analysis (Logan and Edwards, 2009).

In Real-time PCR, cDNA is synthesized from mRNA using reverse transcriptase followed by cDNA PCR amplification and amplicon. The fluorescent signal is quantified using Real-time PCR thermocycler. Real-time RT-PCR reaction contains the same components as in conventional RT-PCR but also has a fluorescent reporter in the form of fluorescent DNA-binding dye or oligonucleotide primer. In amplification, the higher the fluorescence signal, the more it is in proportion to the amount of amplification product. By this, it is possible to identify the exact PCR cycle where the fluorescent signal increases. Precisely measurements of the starting template are provided by this (McPherson and Møller, 2006)

Real-time PCR is important for mRNA gene expression. This is because of its simplicity, high throughput, high speed, and sensitivity and also that small differences can be measured (Vandesompele et al., 2009). The results from the assay depend on the reverse transcriptase and priming that are used to generate cDNA and careful optimization of the PCR step. Accurate data from the assay depend on careful analysis of raw data and the use of normalization. The quality of the RNA template is also important in the RT-PCR assay and should be without DNA and no co-purification of DNA or inhibitors of the RT-step (Bustin and Nolan, 2009).

The data generated in RT-PCR are called crossing thresholds (Ct) and crossing points (CP). They correspond to the number of cycles that are needed to get a defined fluorescence intensity. This intensity is measured in real-time (Guénin *et al*., 2009). Cycle threshold is the PCR cycle number where the signal can be discriminated from the background noise. The greater the amount starting template, the cycle threshold is more rapidly reached, and it gives

lower C_T values. The threshold line defines the C_T value where it intersects with the amplification curve (Stephenson, 2016). Ct values lower than 29 are strong positive reactions by abundant target nucleic acid that is present in the sample. 30-37 are positive reactions by moderate amounts of the target nucleic acid, while 38-40 are weak reactions by minimal amounts of target nucleic acid that could be affected by contamination (WVDL, 2018).

TaqMan gene expression assay is based on 5`nuclease chemistry and is often used for RT-PCR. It uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR. The fluorescent dye is called the reporter and is attached to the 5`end in the probe. On the 3`end in the probe, there is a quencher that absorbs energy from the light that is being used in the dye at the reporter. When both the reporter and quencher are connected, the quencher reduces the fluorescent signal of the reporter dye. Taq DNA polymerase extends the primer on the probe`s target strand through the action of 5`to 3`exonuclease. The reporter dye is released from the quencher and then it fluoresces (Stephenson, 2016).

At the start of the RT-PCR, the temperature is raised to denature the double-stranded cDNA. Here is the signal from the fluorescent on the 5′ end in the TaqMan probe quenched by the Nonfluorescent quencher (NFQ) on the 3′end. The reaction temperature is then lowered where the primers and probe anneal to their specific target sequences. After this Taq DNA polymerase synthesizes a new strand by using the unlabelled primers and the template. When the polymerase gets to a TaqMan probe, the endogenous 5`nuclease activity separates the dye from the quencher. In every cycle of PCR, dye molecules are released and lead to a proportional amount of amplicon synthesized with the increased fluorescence intensity (Scientific, n.d).

Aim

The purpose of this study is to determine the importance of the methylation of PP2A-C done by the regulator LCMT1 under stress conditions in plant cells. Methylation of the C-subunit in PP2A is considered to be significant in cell survival and health under stressed environments. The main objectives are to study *A. thaliana* WT and the knockdown mutant of LCMT1 (*lcmt1*) under stress in the forms of nutrient deficiency and oxidative stress from the addition of H_2O_2. Then to investigate the meaning of methylation of PP2A-C under these conditions.

2. Materials and methods

2.1 Media prepared in Petri dishes

Table 2.1.1: The mixture of ½ Murashige and Skoog (MS) nutrient medium solution.

MS stock solutions	**Mass (g)**
A. KNO_3	95
B. NH_4NO_3	120
C. $MgSO_4$ x $7H_2O$	37
D. KH_2PO_4	17
E. $CaCl_2$ x $2H_20$	44
Fe/EDTA	
Na_2EDTA	0.373
$FeSO_4$ x $7H_2O$	0.278
Minor I	
$ZnSO_4$ x $7H_2O$	0.920
H_3BO_3	0.620
$MnSO_4$ x $4H_2O$	2.230
Minor II	
Na_2MoO_4 x $2H_2O$	0.025
$CuSO_4$ X $5H_2O$	0.003
$CoCl_2$ x $6H_2O$	0.003
KI	0.083

From the Murashige and Skoog procedures shown in Table 2.1.1 (Murashige and Skoog, 1962) the ½ MS nutrients solutions were made, adding 1% sucrose and 0.7% agar.

1/2 of regular Murashige and Skoog (MS) with 1% sucrose media was made by adding 800 mL of distilled water to a measuring beaker, then pipetting the nutrients solutions (Table 2.1.2) into the beaker of the distilled water. 10 g of sucrose was weighed and added to the beaker with a magnet stirrer. The pH of the solution was measured, HCl and KOH were added as required, the preferred value is approximately 5.8. 100-200mL of distilled water was then added to the same beaker. With a measuring cylinder, the solution was transferred to two glass bottles containing 400mL of the solution and one glass bottle containing 200mL of the solution. Agar (2.8 g) was then added to the 400 mL glass bottles and (1.4 g) agar to the 200 mL bottle. The medium was then autoclaved.

Table 2.1.2: The MS medium salts and the quantity used for making ½ MS.

MS medium salts	½ MS
A. KNO_3	10 mL
B. NH_4NO_3	6.5 mL
C. $MgSO_4^3 \times 7H_2O$	5 mL
D. KH_2PO_4	10 mL
E. $CaCl_2 \times 2H_2O$	5 mL
F. Na_2EDTA	25 mL
$FeSO_4 \times 7H_2O$	25mL
Minor I	5 mL
Minor II	5 mL
Water	1000 mL
Sucrose	10 g (1%)
Agar	7 g (0.7%)

The other media prepared for the analysis was b) Exclude KNO_3 and exclude NH_4NO_3; c) Exclude KH_2PO_4; d) Exclude KNO_3 and change KH_2PO_4 with NaH_2PO_4. The different media was prepared with the same method as ½ MS media and then autoclaved. Furthermore, approximately 25 mL of the different media was poured into separate square Petri dishes.

2.2 Sterilization of Arabidopsis seeds

Table 2.2.1. Chemicals used for the sterilization of Arabidopsis seeds

Chemicals
Ca-hypochlorite (0.25g)
Tween (1 drop)
95% ethanol (11 mL)

Ca-hypochlorite (0.25g) was added to distilled water (25 mL) in a measuring cylinder. Then 1 drop of Tween was added to make 1% Ca-hypochlorite. This solution was then shaken and left to settle. After a few minutes, the solution (1 mL, supernatant) was pipetted into ethanol (95%, 9 mL). To sterilize the seeds, they were placed into Eppendorf tubes (0.1 mL). The

solution with ethanol and hypochlorite was added to the tubes. Then the tubes were shaken and left incubating for 4 minutes. Further, the supernatant was pipetted off.
Ethanol (95%, 1 mL) was then pipetted to the tubes, and the tubes were shaken. This washing was repeated two times, and it was made sure that the ethanol was well removed each time. After this, the seeds were left to dry in a sterile hood for 4-5 hours and then sealed with parafilm.

2.3 WT and LCMT1 Arabidopsis seeds in Petri dishes

Sterilized WT and lcmt1 Arabidopsis seeds in Eppendorf tube were transferred using a sterilized toothpick and then sown on ½ MS media in square Petri dishes in a sterile hood. Two parallels of WT and lcmt1 were sown, 50 seeds were sown in each of the two lines in both parallels. First, the Petri dishes were placed in a dark room for 2-3 days with a temperature of 4°C. Then moved to a 16-h light/ 8-h dark cycle with a temperature of 22°C for 5-7 days.

Further, the seedlings then were transferred to the different stress media b), c) and d) and also a control media (MS). Five WT and five lcmt1 were moved to square Petri dishes with sterile tweezers in a sterile hood to 3 parallels of each stress media and 3 parallels of the control media. The Petri dishes were then placed in the 16-h light/ 8-h dark cycle for 5-7 days.

2.4 Anthocyanins

WT and *lcmt1* rosette leaves from the stress media without KH_2PO_4 were checked for anthocyanin levels. The shoots were cut from the root using a scalpel blade. Plant material of 0.05 g was collected, two parallels from WT and two parallels from lcmt1 to have adequate weight. The rosette leaves were then transferred to separate Eppendorf tubes. The plant material was extracted with 300 µl methanol, with 1% HCl. The Eppendorf tubes were incubated at 4°C with constant shaking overnight. After the incubation, the extracts were mixed with 200 µl distilled water and 500 µl chloroform in a sterile hood. The solution was then centrifuged for 2 min at 13.000 rpm.

The purple-pink upper layer was then pipetted and added to new Eppendorf tubes, and 600 µl 1% HCl-methanol mixture was added. The solution was shaken well before a new round of centrifugation at 13.000 rpm for 2 min. Following this, the extract was transferred to a cuvette and measured with a spectrophotometer with the absorbance at 535 and 657 nm. From the

absorbance the relative concentration of the anthocyanins can be calculated with the following equation:

$$(1)\ Relative\ C_{anthocyanins} = \frac{(absorbance\ at\ 535nm) - (absorbance\ at\ 657nm)}{plant\ material\ (g)}$$

2.5 Oxidative stress

Refrigerated 30 % H_2O_2 9.8M (0.5 mL) was pipetted into an Eppendorf tube, then put inside the sterile hood. Standard ½ MS medium with 1% sucrose (12 mL) was poured into a Falcon tube, following (0.5 mM) H_2O_2 was pipetted into the tube. The tube was gently turned to mix. Additionally, MS was filled to the 25 mL mark. The 25 mL was poured into a round Petri dish. Round Petri dishes with just MS (0 mM H_2O_2) were used as a control medium. The seedlings are transferred with a sterile tweezer from Petri dishes of ½ MS media that had been placed in a 16-h light/8-h dark room with a temperature of 22°C for 5-7 days. Seedlings of five WT and five lcmt1 in each Petri dish. The dishes were then secured with parafilm and placed in a 16-h light/ 8-h dark room with a temperature of 22°C for 5 days. Subsequently, plants are sealed in aluminium foil then froze at -70ºC for further gene analyses.

2.6 Purification of Total RNA from plant cells

After weighing both the A. Thaliana grown in Petri dishes with ½ MS and with H_2O_2 (0.5 μL), they were placed on aluminium foil and then packed together. The aluminium foil was then marked and put in liquid nitrogen (N_2) before they were placed in a -70ºC freezer. It was important to work quickly so that the plant material didn't dry out while weighing. For the RNA extraction of the plant materials, it was used the PureLink$_{TM}$ RNA Mini Kit (Thermo Fischer Scientific, Inc, Waltham, MA, USA).

2.6.1 Preparations

Before starting on the RNA extraction β-Mercaptoethanol (β-ME) needed to be added to Buffer RLC (lysis buffer), where β-ME (10 μL) was added per 1 mL Buffer RLC. Four samples of a maximum of 100 mg required 2 mL lysis buffer and therefore 20 μL β-ME was added. This was prepared in a fume hood because guanidine salt in the buffer and β-ME are toxic. The buffer that was added to β-ME is stored at room temperature for a maximum of

one month. Buffer RPE was supplied as a concentrate and therefore 4 volumes of ethanol (96-100%) were added.

2.6.2 Preparations before DNase treatment

Lyophilized DNase (1500 Kunitz units) was dissolved in RNase-free water (550 μL) that was provided. Then it was mixed gently. For long-term storage of DNase I, the stock solution could be removed from the glass vial and divided into single-use aliquots and then placed in -20ºC for up to 9 months. For short-time storage the DNase I was stored at 2-8ºC for up to 6 weeks. In this experiment, 500 μL of lysis buffer was required for a sample of a minimum of 80 to a maximum of 100 mg. DNase I (10 μL) was added to buffer RDD (70 μL) to make the DNase I incubation mix (80 μL). It was mixed by gently inverting the tube. Then the mixture was centrifuged briefly.

2.6.3 Procedure

Buffer RLC (450 μL) was added to a maximum of 100 mg of the samples in the Eppendorf tubes. Then the samples were vortexed vigorously before incubated at 58ºC to help disrupt the tissue. The lysate was then transferred to a QIAshredder spin column (lilac) that was placed in a 2 mL collection tube and centrifuged for 1 min at 13.000 rpm. Then the QIAshredder spin column was removed, and the supernatant of the flow-through in the collection tube was carefully pipetted to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. Only the supernatant was used in some of the next steps.

0.5 volume of ethanol (225 $\mu L, 95\%$) was added to the cleared lysate and mixed by pipetting. The samples (approximately 650 μL) including any precipitate were transferred to a RNeasy spin column (pink) placed in a 2 mL collection tube. The lid was closed gently and centrifuged for 1 min and then the flow-through was discarded. The RNeasy was then placed into a new collection tube.

To remove the genomic DNA 350 μL Buffer RW1 was added to the RNeasy spin column, and then centrifuged for 1 min at 13.000 rpm. The flow-through was discarded. The RNeasy was then placed into a new collection tube again. DNase I incubation mix (80 μL) was added directly to the RNeasy spin column membrane, and then placed on the bench at room temperature for 15 min. After 15 min 350 μL buffer RW1 was added to the column and

centrifuged for 1 min at 13.000 rpm. The flow-through was discarded, but the collection tube was reused in the next step. Buffer RW1 (700 μL) was added to the column and centrifuged again for 15 sec at 13.000 rpm. The flow-through was discarded, and the collection tube was reused in the next step. Then the same procedure was done again, but with RPE buffer (500 μL) instead of buffer RW1 and centrifuged for one minute. The flow-through was discarded, and the collection tube was reused in the next step. Repeated the same again with 500 μL RPE, but this time it was centrifuged for 2 min. The flow- through and the collection tube were discarded.

The RNeasy spin column was then placed in a new 2 mL collection tube and the sample was centrifuged at full speed for two minutes at 13.000 rpm. Again, the column was placed in a new 1.5 mL collection tube. RNase-free water (25 μL) was added and then incubated on the benchtop for 2 min before centrifuged for 2 minutes at 13.000 rpm. This was done twice. This time the flow-through is kept and used in Nanodrop to see how pure the RNA was.

2.7 cDNA synthesis

2.7.1 Preparing the cDNA RT reactions:

Used the Applied Biosystems™ High-Capacity RNA-to-cDNA™ kit. (Thermo Fischer Scientific, Carlsbad, CA, USA). First, the kit components thawed on ice then the RT reaction mix was prepared (per 100 μL reaction).

Table 2.7.1: RT reaction mix components for one of the four Eppendorf tubes.

Component	**Volume (µL)**
10 x RT buffer	10.0
25 x DNTP Mix (100 mM)	4.0
10 x RT Random Primers	10.0
Multiscribe™ Reverse Transcriptase	5.0
RNase inhibitor	5.0
Nuclease-free water	16.0
Total per Reaction	**50.0**

Table 2.7.2: Nuclease free water and RNA sample (50 µL) used from WT and *lcmt1* in media containing ½ MS (0 mM) or 0.5 mM H_2O_2 to make cDNA determined from Nanodrop values.

	WT_0	$lcmt1_0$	$WT_{0.5}$	$lcmt1_{0.5}$
RNA sample (μL)	6.6	9.1	7.0	6.0
Nuclease free H_2O (μL)	43.4	40.9	43.0	44.0

Nuclease free water and RNA template together had a volume of 50 μL. The master mix was added first before Nuclease-free water and RNA sample. Together it had a total volume of 100 μL that were needed in each of the four Eppendorf tubes that was going to be used. How much of template RNA that was added where determined from the results of Nanodrop. This was then transferred to four small Eppendorf tubes with 100 μL in each. Then the tubes where sealed and briefly centrifuged to eliminate air bubbles and after this the tubes where placed on ice until one was ready to use the thermal cycler.

2.8 Performing the reverse transcription

Table 2.8.1: Reverse transcription program in the thermal cycler.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature(°C)	25	37	85	4
Time(min)	10	120	5	∞

The reaction volume was set to 100 μL and the reaction was loaded into the thermal cycler and then started the reverse transcription. After the time in the thermal cycler, the cDNA was stored at -70°C for long time storage.

2.9 Real-time PCR (Gene analysis)

Target amplification is the second step in the RT-PCR experiment using cDNA. TaqMan® Universal Master mix II is used in the PCR analysis. (Applied Biosystems by Thermo Fischer Scientific, Vilnius, Lithuania). PCR is a technique important for mRNA gene expression where small differences can be measured (Vandesompele *et al.,* 2009). The RNA template should be without DNA and no inhibitors in the RT-step to give better quality (Bustin and Nolan, 2009). The RT-PCR analysis was performed using LightCycler® 96 instrument that is used for rapid cycling up to 96 samples. By performing this instrument, it can show the absolute and relative quantification, melting curve analysis, and endpoint genotyping. Multiplex and multicolor give the use for up to four different fluorescent dyes (Life science, 2018).

Table 2.9.1 Components added to the PCR wells

Component	**Volume (μL)**
Taqman® Universal Master Mix II	7
Taqman® Gene expression assay	1
cDNA template + Nuclease-free H_2O	12
Total volume	20

cDNA (2.5 μL) and nuclease-free water (9.5 μL) together with the master mix and gene expression assay (8 μL) were pipetted into a MicroAmp® Fast optical 96-Well Reaction Plate used from Applied Biosystem™ (Carlsbad, CA, USA). Some of the wells did not contain cDNA, only RNA, and were used as a control well. The plate was covered with MicroAmp® Optical Adhesive film and then the plate was centrifuged to eliminate the air bubbles. Then it was placed into the PCR-machine with the parameters in table 2.9.2.

Table 2.9.2 Parameters in the PCR machine (Applied Biosystem, 2010)

System	**Polymerase activation**	**PCR**	
	Hold	**Cycle (40 cycles)**	
		Denature	**Anneal/extend**
Temp (ºC)	95	95	60
Time (min: sec)	10:00	00:15	1:00

Three genes were tested from the plants that grew with or without 0.5 mM H_2O_2. The genes were *FSD1*, *APXs,* and *HSP90*. Other genes that were used as intern control were *UBC35, UBQ,* and *ACT8.*

Table 2.9.3: TaqMan assay in the Real time-PCR analysis of the genes that were used. The reference genes *UBQ, UBC35* and *ACT 8* were used as intern control.

Gene	**Accession number**	**Taqman ID**
FSD1, Fe-superoxide dismutase 1	At4g25100	At02238153_ g1 Catalog nr: 4351372
sAPX, Ascorbate peroxidases	At4g08390	At02210256 _ g1 Catalog nr: 4351372
HSP90-1, Heat Shock Protein 90-1	At5g52640	At02320696 _ g1 Catalog nr: 4351372
UBC35, UBIQUITIN CONJUGATING ENZYME 35	At1g78870	At02612351 _ g1 Catalog nr: 4351372
UBQ10, UBIQUITIN 10	At4g05320	At02353386_ s1 Catalog nr: 4351372
ACT8, Actin 8	At1g49240	At02270958 _ gH Catalog nr: 4331182

3. Results

3.1 Experiment 1: Arabidopsis; root length and plant weight.

A. thaliana WT and mutant *lcmt1* seedlings were placed in media containing ½ MS and different stress media. Originally there were three Petri dishes of each media containing ½ MS, -N, -P, and -K, but some of them were contaminated and were not used to compare with the others.

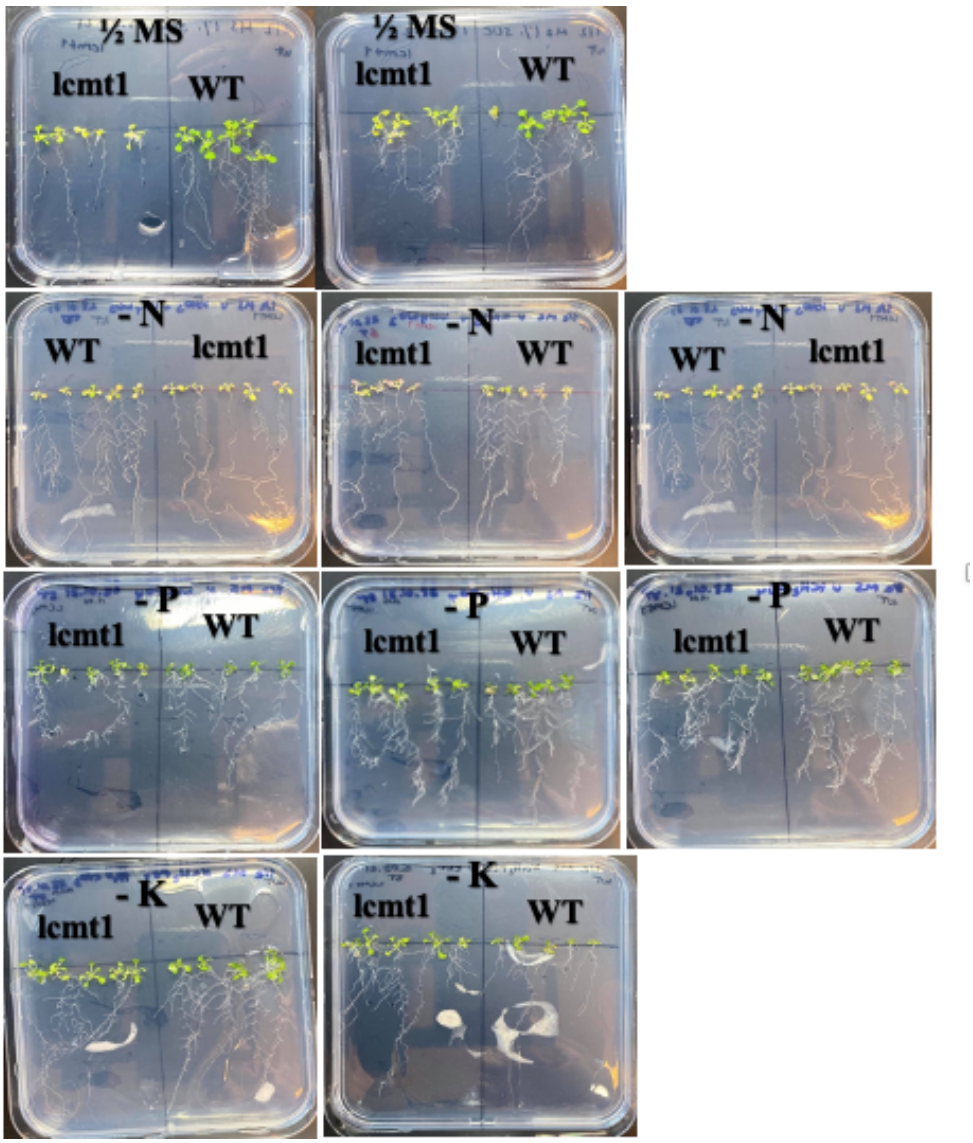

Figure 3.1.1: The first parallel of experiment 1. Five WT and five *lcmt1* seedlings placed on Petri dishes. First the seedlings were placed in ½ MS for 3 days in the dark and 5 days in 16-h-light/ 8-h dark cycle. After 8 days WT and *lcmt1* were moved to different media and then placed back for 7 days in 16-h light/ 8-h dark cycle. The medium ½ MS with 1% sucrose as control medium, ½ MS with 1% sucrose without KNO_3 and NH_4NO_3, ½ MS with 1% sucrose without KH_2PO_4 and ½ MS with 1% sucrose with NaH_2PO_4, without KNO_3. The red dot shows the root elongation after the seedlings were placed on different medium. The result of the root length was measured with ImageJ.

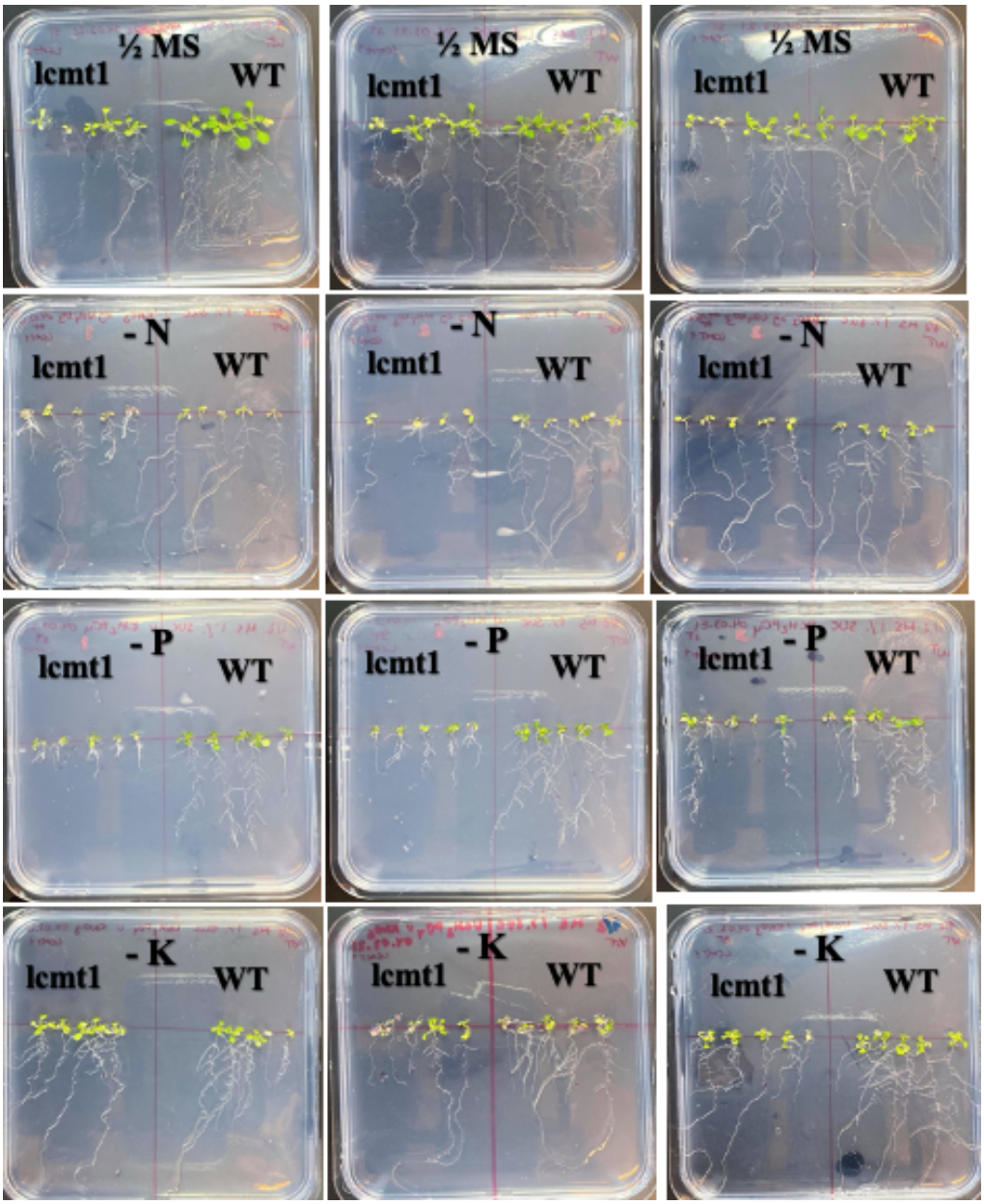

Figure 3.1.2: The second parallel of experiment 1. Five WT and five *lcmt1* seedlings placed on Petri dishes. First the seedlings where placed in ½ MS for 3 days in the dark and 5 days in 16-h light and 8-h dark cycle. After 8 days WT and *lcmt1* were moved to different media and then placed back for 7 days in 16-h light/ 8-h cycle. The medium ½ MS with 1% sucrose as control medium, ½ MS with 1% sucrose without KNO_3 and NH_4NO_3, ½ MS with 1% sucrose without KH_2PO_4 and ½ MS with 1% sucrose with NaH_2PO_4, without KNO_3. The red dot shows the root elongation after the seedlings were placed on different medium. The result of the root length was measured with ImageJ.

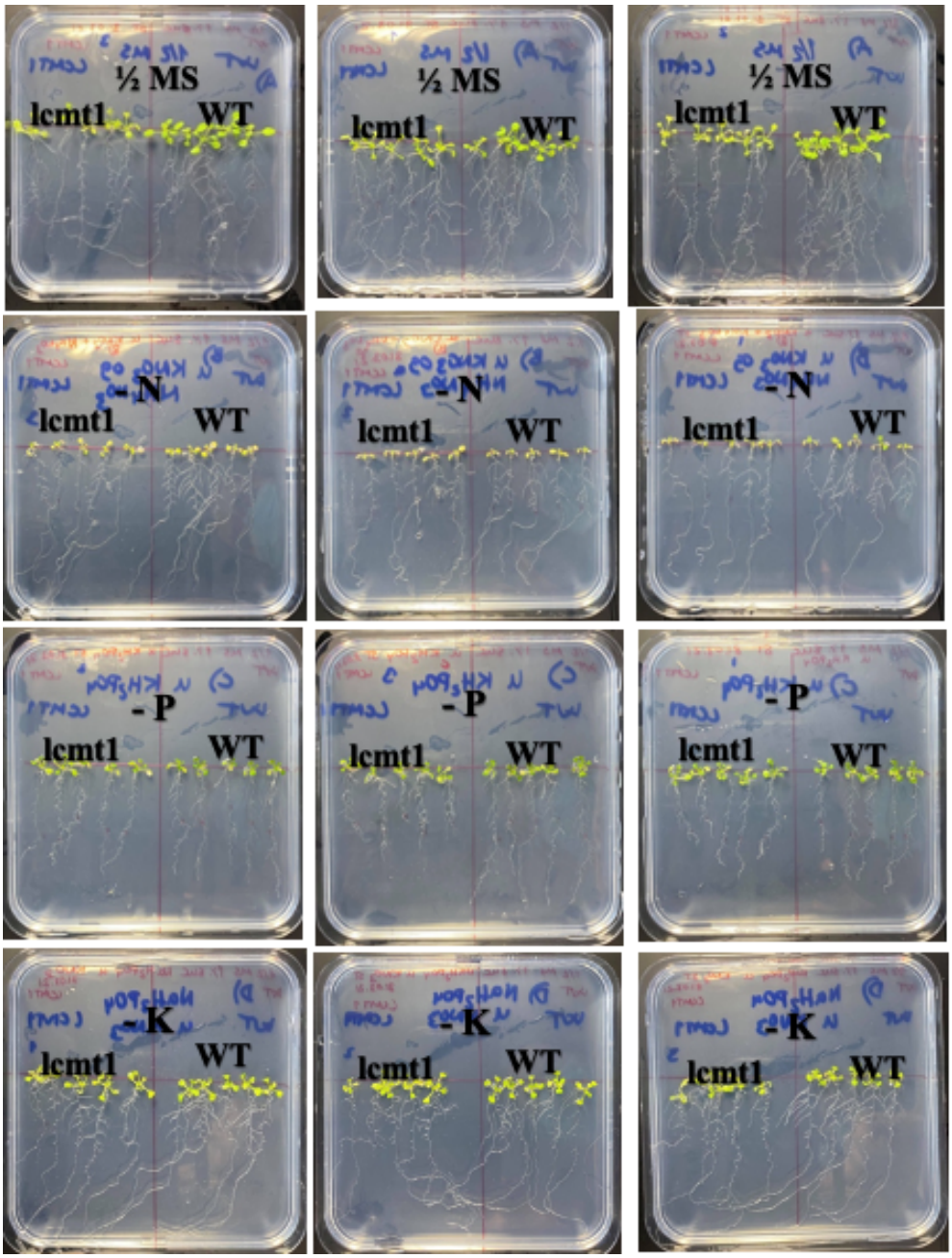

Figure 3.1.3: The third parallel of experiment 1. Five WT and five *lcmt1* seedlings placed on Petri dishes. First the seedlings where placed in ½ MS for 3 days in the dark and 6 days in 16-h light and 8-h dark cycle. After 9 days WT and *lcmt1* were moved to different media and then placed back for 7 days in 16-h light/ 8-h dark cycle. The medium ½ MS with 1% sucrose as control medium, ½ MS with 1% sucrose without KNO_3 and NH_4NO_3, ½ MS with 1% sucrose without KH_2PO_4 and ½ MS with 1% sucrose with NaH_2PO_4, without KNO_3. The red dot shows the root elongation after the seedlings were placed on different medium. The result of the root length was measured with ImageJ.

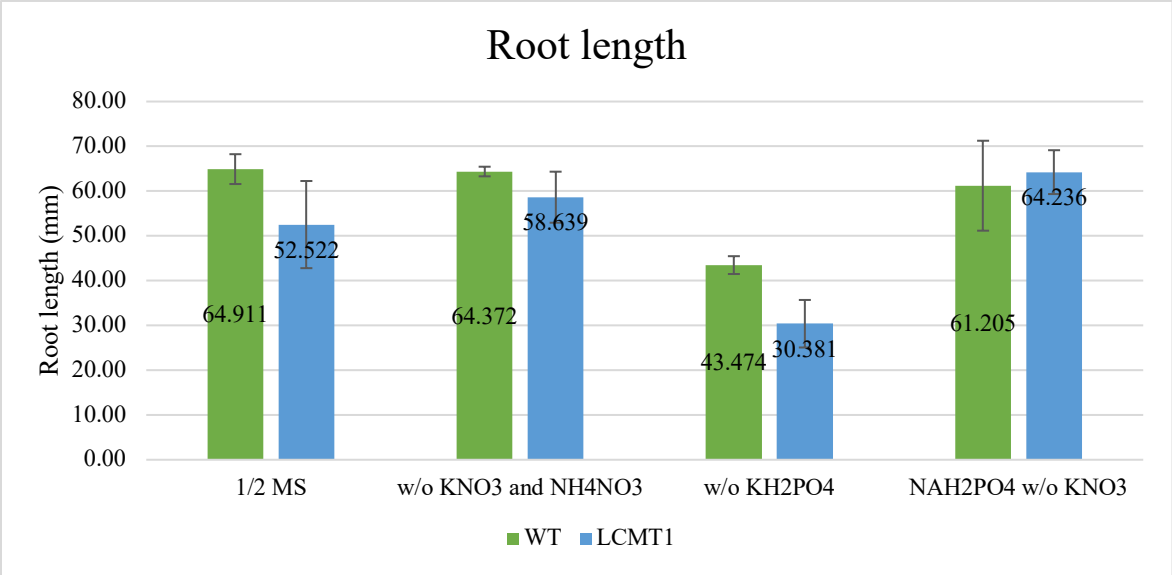

Figure 3.1.4: The root length of every plant of *A. thaliana* WT and *lcmt1* was measured by using ImageJ. Plants were measured after seven days of growth in 16-h light/8-h dark cycle on three different stress media and one control media (1/2 MS). P-values from three parallels of all treatments were >0.05 and *lcmt1* were not significantly different from WT. SE is presented in the vertical bars.

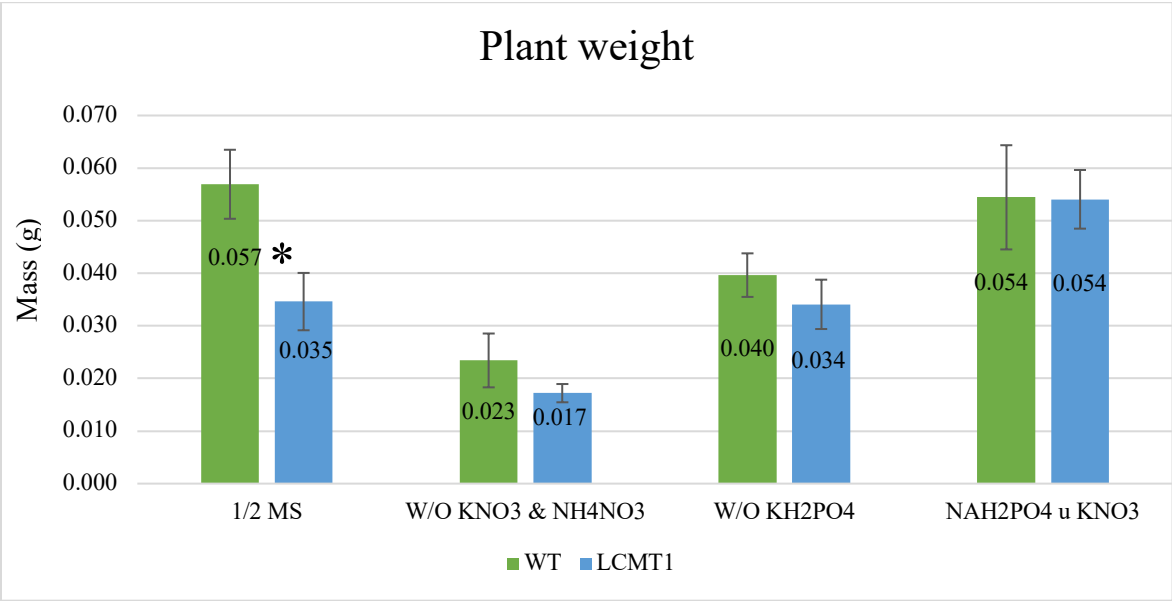

Figure 3.1.5: The average plant weight of five WT and *lcmt1* in different Petri dishes measured after 7 days of growth threatened with different media, one containing all of the nutrients, one without KNO_3 and NH_4NO_3, one without KH_2PO_4, and one with NaH_2PO_4 instead of KH_2PO_4 and without KNO_3. The plants were grown for 7 days in a 16-h light/8-h dark cycle. SE is given as vertical bars and p values < 0.05 are denoted with *.

In the second parallel the deficiency of potassium and nitrate the mutant *lmct1* indicated some anthocyanin production. The third parallel showed the best results; however, it was difficult to distinguish which of WT or the knockdown *lcmt1* responded best to the various stress treatment by just observing them. Since the results were so different from parallel to parallel. By weighing them and then measuring them using ImageJ, WT on average had more elongated roots except in the stress media without nitrate and potassium and added sodium phosphate. WT had larger shoots and weighed more in comparison to *lcmt1* in all treatments. Both WT and *lcmt1* grew root hairs especially long in the control media of ½ MS with 1% sucrose, but also in the stress treatments. In *Arabidopsis thaliana,* root hair is on average 10 μm in diameter, and they can elongate to 1 mm or even more. Root hair increases the surface area and the diameter of the root. Research has suggested that the root hair helps the plant in accumulating nutrients, the microbe in interactions, and also in anchorage (Grierson *et al*.,2014). Overall the visible phenotypes of WT and *lcmt1* seemed to react similarly in the treatments done in this study.

3.2 Anthocyanins

Table 3.2.1 Anthocyanin concentration in shoots of Arabidopsis WT and *lcmt1* after growth on media without KNO_3 and NH_4NO_3. Plants from the second parallel of experiment 1 were used in this study. Results were calculated by the formula absorbance at 530- absorbance at 657 divided by the mass of the tissue sample.

Tissue sample	**Mass(g)**	**Absorbance at 530nm**	**Absorbance at 657nm**	**Relative concentration(l/g)**
WT	0.047	0.620	0.020	12.80
lcmt1	0.025	0.085	0.009	3.040

In medium ½ MS without KNO_3 and NH_4NO_3, all nitrate is eliminated, which normally acts as a suppressor for anthocyanins synthesis. Flavonoids such as anthocyanins are produced in plant tissues as a reaction to abiotic stress such as too much light, drought or nitrogen deficiency, etc. Anthocyanins are linked to giving plants a higher stress tolerance, may function as a ROS scavenger. Researchers have suggested that in *A. thaliana* anthocyanins have various roles in different kinds of abiotic stresses (Kovinich *et al*., 2015).

In our study, the seedlings of WT did not necessarily grow longer roots than *lcmt1* on medium without nitrate, but the shoots weighed more in the WT phenotype. For this reason, the mass

of mutant *lcmt1* was barely half of WT´s mass in this experiment. Ideally, both plant tissues should have been at least 0.05 g for the most accurate result. The relative concentration of anthocyanins in WT was calculated to 12.80 and was 4 times higher than the relative concentration value in *lcmt1*. The shoot weight difference needs to bear in mind when evaluating the relative concentration result. WT may have higher levels of anthocyanins induced in stress conditions in contrast to *lcmt1*.

3.3 Oxidative stress induced from H_2O_2

3.3.1 Plant weight measurement of different concentrations of H_2O_2

Five WT and five lcmt1 per Petri dish were measured after treatment with and without H_2O_2. 3 replicates from ½ MS media and different H_2O_2 media (1 mM, 1.5 mM, 2.5 mM and 3.75 mM) were needed to increase the reliability of the results. A bar diagram (Figure 3.3.1) was made of the average plant weight from the different treatments. Figure 7.2.1 in appendix shows the first failed attempt with high concentrations of H_2O_2.

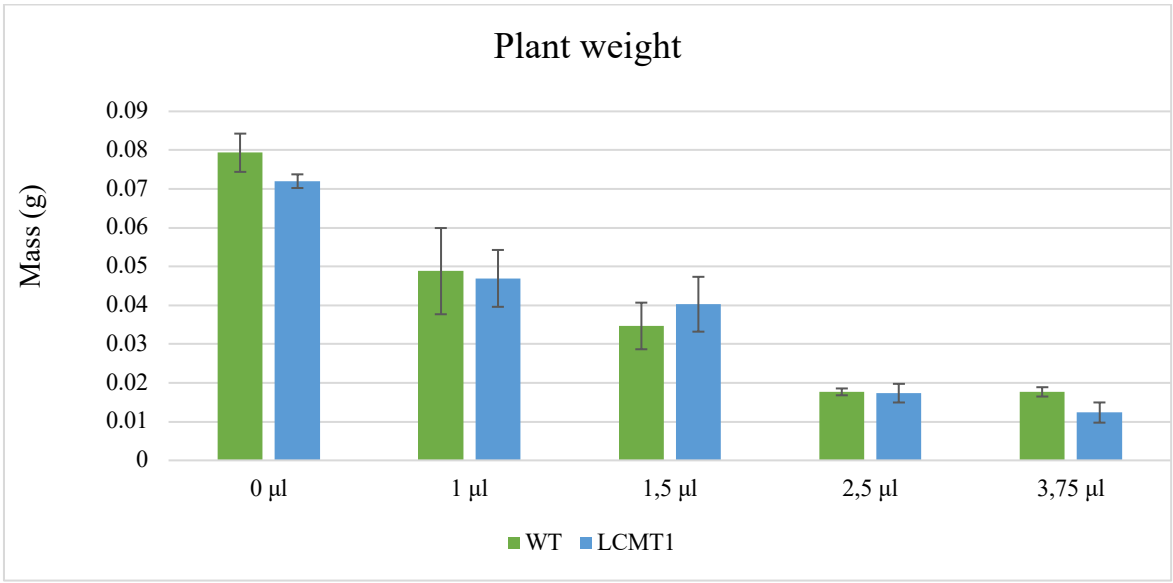

Figure 3.3.1. The first parallel of the experiment with H_2O_2. The average plant weight of five WT and five *lcmt1* from three Petri dishes of each five treatments (0 mM, 1 mM, 1.5 mM, 2.5 mM, and 3.75 mM H_2O_2) placed in a 16h light/8h dark cycle. The plants were grown in ½ MS media for 2 days in dark and 5 days in 16-h light/ 8 h dark cycle. The plants were then treated with the different stress media and were placed back in the 16-h light/8-h dark cycle for 6 days. SE is given as vertical bars. All treatments had p-values>0.05 and therefore *lcmt1* is not considered significantly different from WT. However, the growth of the plants in the control medium was also impaired, this needs to be taken into consideration when viewing the results of *A. thaliana* growth in high concentrations of H_2O_2.

The first experiment with oxidative stress was not successful, because of almost no growth. The root elongation was inhibited because the concentrations were too high and there was no difference in the root length before and after the plants were placed in the high concentration media. To show the interesting effects it was necessary to lower the concentrations. Figure 3.3.1 shows that the plant weight of both WT and *lcmt1* were affected by the high concentrations. The higher concentrations, the more decreasing weight.

3.3.2 Root length and plant weight measurement of lowered concentration of H_2O_2

The same procedure was done for the experiment with 0 mM and 0.5 mM H_2O_2. This experiment was performed two times, and the root length and plant weight were measured using the mean of all the parallels in both of the experiments. Two bar diagrams (Figure. 3.3.6 and 3.3.7) were made from the average root length and plant weight. The root length measurements of five WT and five *lcmt1* per Petri dishes were done by using ImageJ.

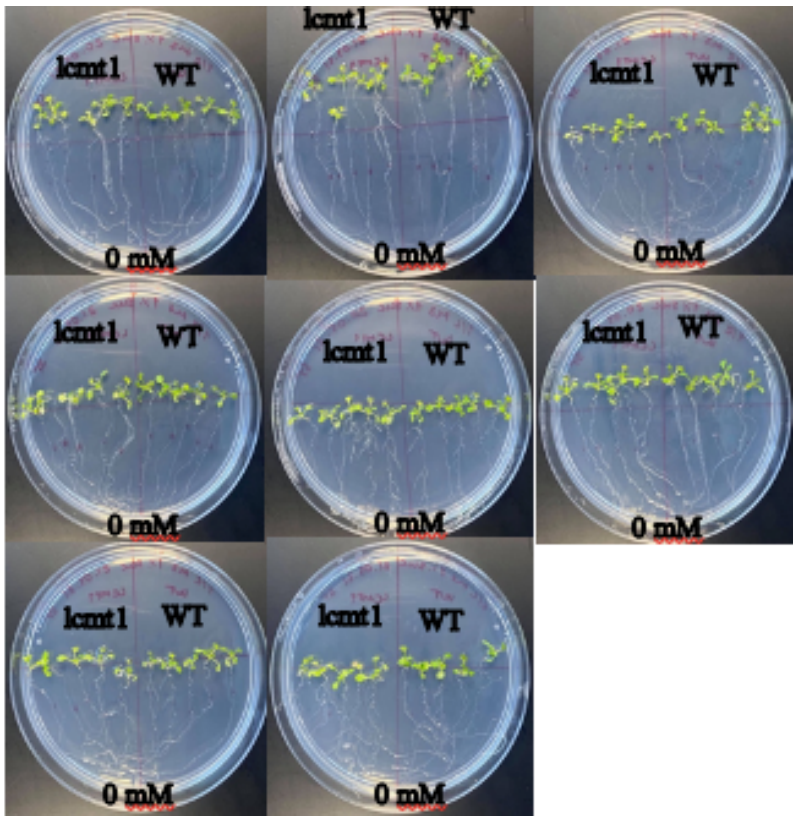

Figure 3.3.2. The second parallel of experiment 2. Growth of *A. thaliana*, five WT and five *lcmt1* in control medium ½ MS 1% sucrose, 0 mM H_2O_2. The seedlings were grown in ½ MS media for 2 days in dark and 5 days in 16-h light /8-h dark cycle. After 7 days the plants were treated with ½ MS media and placed back for 6 days in the 16-h light/ 8-h dark cycle. Eight replicates of *lcmt1* and WT were

made (40 WT and 40 lcmt1). The root length after the red mark shows the root elongation after the seedlings were placed in the 16-h light/8-h dark cycle for 5-days. The elongation was measured by using ImageJ.

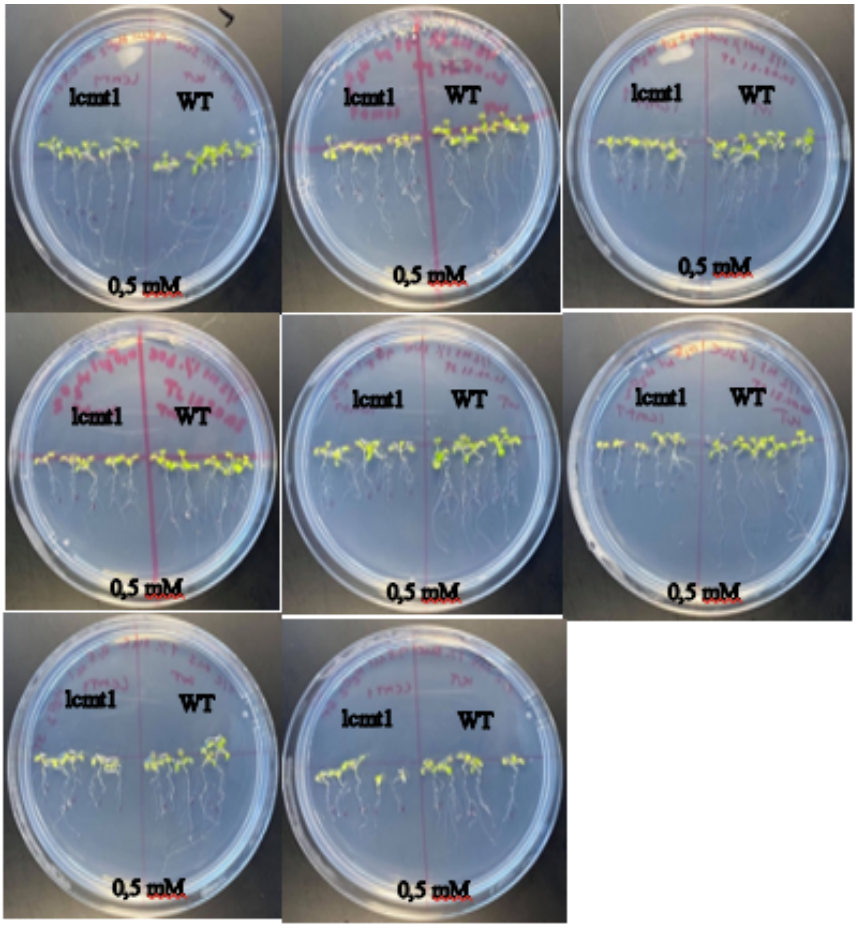

Figure 3.3.3. The second parallel of experiment 2. *A. thaliana*, five WT and five *lcmt1* seeds placed on Petri dishes containing ½ MS 1% sucrose with 0.5 mM H_2O_2. The seedlings were grown in ½ MS media for 3 days in dark and 5 days in 16-h light/8-h dark cycle. After 8 days the plants were treated with 0.5 mM H_2O_2 media and placed back for 6 days in 16-h light/ 8-h dark cycle. Eight replicates of lcmt1 and WT were made (40 WT and 40 lcmt1). The root length after the red mark shows the root elongation after the seedlings were placed in the 16-h light/8-h dark cycle for 5-days. The elongation was measured by using ImageJ.

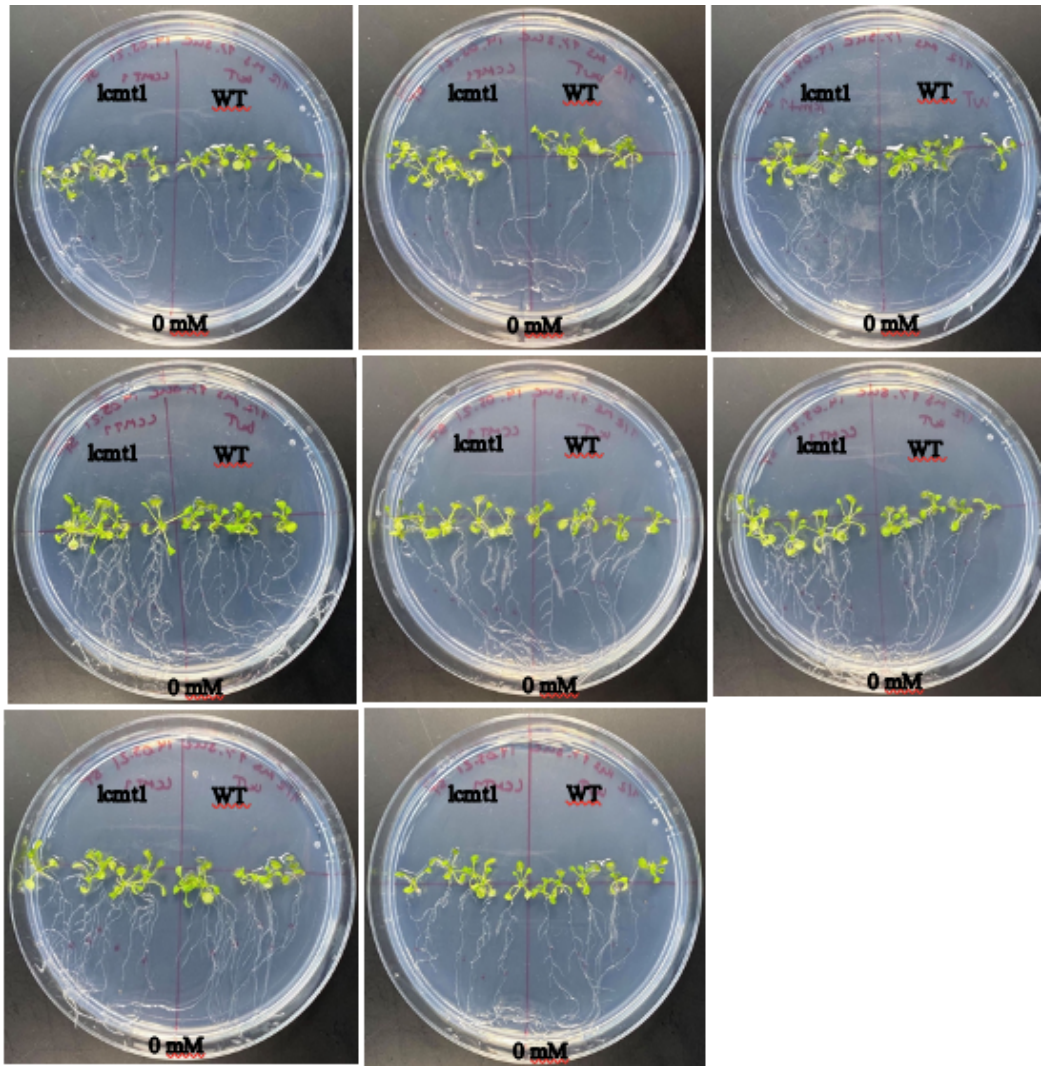

Figure 3.3.4: The third parallel of experiment 2. Five WT and five *lcmt1 A. thaliana* seedling growth of 6 days in 16-h light/8-h dark cycle on control medium ½ MS with 1% sucrose. The seedlings were grown in ½ MS media and placed in the dark for 3 days and then 5 days in a 16-h light / 8-h dark cycle. After 8 days the plants were treated with ½ MS media and placed back for 6 days in 16-h light / 8 h-dark cycle. Eight replicates of *lcmt1* and WT were made (40 WT and 40 lcmt1). The root length after the red mark shows the root elongation after the seedlings were placed in the 16-h light/8-h dark cycle for 5-days. The elongation was measured by using ImageJ.

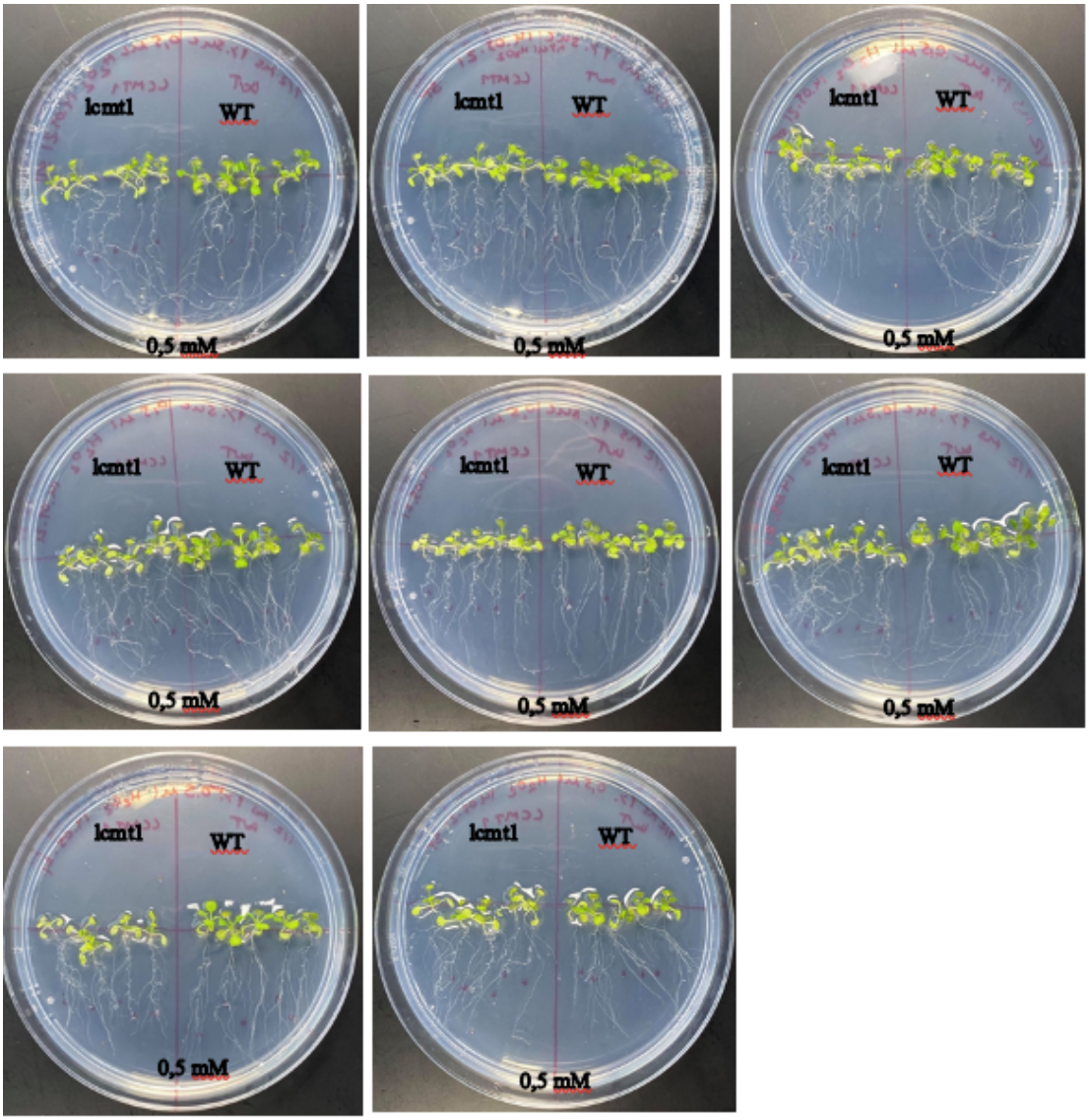

Figure 3.3.5: The third parallel of H_2O_2 experiment, five WT and five *lcmt1 A. thaliana* seedling placed on 8 Petri dishes containing 0.5 mM H_2O_2 pipetted to ½ MS with 1% sucrose. The seedlings were grown in ½ MS media for 3 days in dark and 5 days in a 16-h light / 8-h dark cycle. After 8 days the plants were treated with 0.5 mM H_2O_2 media and placed back for 6 days in a 16-h light/ 8-h dark cycle. The root length after the red mark shows the root elongation after the seedlings were placed in the 16-h light/8-h dark cycle for 5-days. The elongation was measured by using ImageJ.

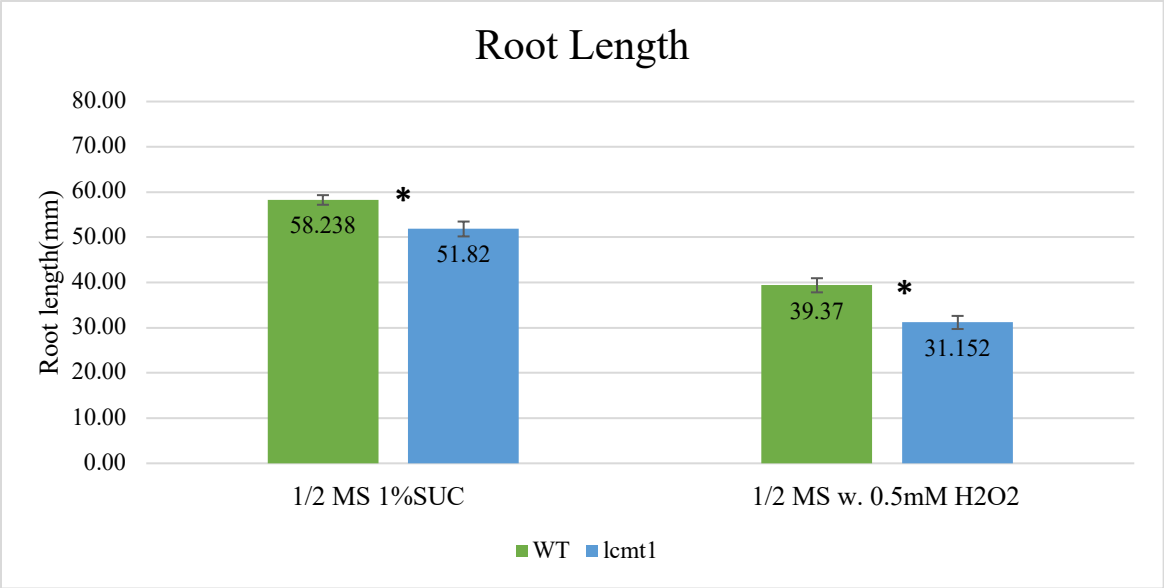

Figure 3.3.6: The average root growth of *A. thaliana* on control media ½ MS 1% sucrose and on 0.5 mM H_2O_2, measured from pictures of the last two parallels using ImageJ software. The WT and *lcmt1* seeds were grown in ½ MS media for 7 days (2 days in dark and 5 days in 16-h light/8-h dark cycle). Then the plants were treated with control (½ MS, 0 mM H_2O_2) and 0.5 mM H_2O_2. The roots were measured after 5 days in a 16-h light/8-h dark cycle. p-values<0.05 is symbolized with *, shows when the mutant *lcmt1* significantly different from WT. SE is revealed in the vertical bars.

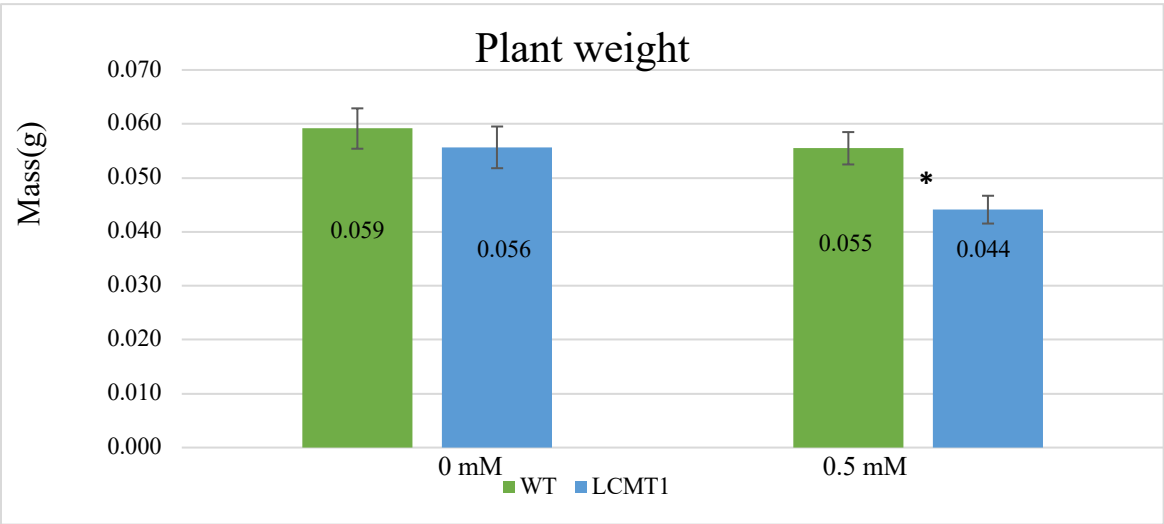

Figure 3.3.7. The average plant weight of WT and *lcmt1* seedlings in Petri dishes with ½ MS (0 mM) and 0.5 mM H_2O_2.The seedlings were placed in ½ MS media for 2 days in the dark and 5 days at 16-h light / 8-h dark cycle. Then the plants were placed in Petri dishes containing media of 1/2 MS, 0 mM H_2O_2 or 0.5 mM H_2O_2. The plants were measured after 7 days of growth in a 16-h light/ 8-h dark cycle. SE is given as vertical bars. The plant weight of mutant *lcmt1* decreases more than WT when 0.5 mM H_2O_2 is added to the samples. The plant weight of WT did not seem impaired by the addition

of 0.5 mM H_2O_2 compared to the control (0 mM). p-value 0.0062<0.05 in the stressed treatment, which means the mutant *lcmt1* is significantly different from WT.

The concentration of H_2O_2 was lowered to 0.5 mM because we needed more visible phenotypical traits of the plants for further studies. Figure 3.3.7 showed that the oxidative stress indicated the highest inhibition on *lcmt1* compared to WT.

Oxidative stress is simulated by inserting H_2O_2 to ½ MS with 1% sucrose medium and this induces the formation of toxic ROS in plant tissues (Claeys *et al*., 2014). From analyzing the samples of both parallels, the addition of 0.5 mM H_2O_2 to the standard medium of ½ MS with 1% sucrose showed to had quite a large effect on each phenotype. In terms of root growth, oxidative stress indicated the highest inhibition on *lcmt1*. The induced oxidative stress stunted the growth of *lcmt1*, both the weight and the root development were affected. On average, WT grew longer roots in comparison to *lcmt1*. The shoots of WT were more elongated and appeared greener than the knockdown *lcmt1*. In terms of average plant weight, the weight for both WT and lcmt1 decreased with an increasing concentration of H_2O_2. *lcmt1* treated with 0.5 mM H_2O_2 was more inhibited compared to WT in the same media. The control (1/2 MS) indicated a lower weight of *lcmt1* than WT as well. The P-value was below 0.05 for 0.5 mM H_2O_2 and this showed that *lcmt1* was significantly different from WT.

3.4 RNA concentration

Table 3.4.1: Concentration of the RNA from the plant tissue samples WT-0, WT-0.5, *lcmt1-0*, and *lcmt1-0.5*. Results from the Nanodrop 2000. The ratio of A260/A280 had values over 2, which indicates that the RNA was pure. Ratio A260/A230 was a bit higher, this can occur for several reasons.

Sample	**RNA**	**Concentration(ng/mL)**	**Ratio of absorbance at (260/280nm)**	**Ratio of absorbance at (260/230nm)**
1	WT-0	1527.3	2.15	2.43
2	WT-0.5	1436.1	2.15	2.40
3	lCMT1-0	1103.1	2.11	2.37
4	lCMT1-0.5	1662.6	2.14	2.42

Examination of RNA concentration from the four samples was measured with the ratio of absorbance at 260/280 nm. This absorbance ratio is used to find the purity of DNA and RNA. For RNA a ratio of ~2.0 indicates an uncontaminated RNA sample. The ratio of absorbance at 260nm and 280nm WT and *lcmt1* both have values < 2 between 2.0-2.15 and are of great quality. The samples are not considered polluted. The second ratio of 260/230nm also measures the RNA purity, values here tend to be higher than the first ratio, approximately ~2.0-2.2 is usual (Scientific, 2010).

Before the concentration of samples was measured on the Nanodrop, the plants were placed on Petri dishes for 6-days in a 16-h light/8-h dark cycle. The plants were then moved to eight parallels of each medium, one control, and one stress-induced with 0.5mM H_2O_2. Then placed in a 16-h light/8-h dark cycle for 6-days, after the plants were harvested and frozen at -70°C.

The RNA isolation of the plant samples was made from freezing them in liquid nitrogen and then grounding them with a mortar and transfer the material to four Eppendorf tubes for the four different samples, 1 control and 1 stress condition for each phenotype WT and *lcmt1*. The RNA samples (0.5 mL) are then pipetted on the measurement pedestal. Inside this pedestal, there is a fiber optic cable. Supplementary there is a second fiber optic cable, that is in contact with the fluid, and then creating a connection between two fibers` ends. The light

source is a pulsed xenon flash lamp, it also holds a spectrometer that uses linear CCD array analysis (Scientific, 2009).

Results from the ratio of absorbance at 260 and 230 nm are a tad high and this can occur for several reasons. An unusual value at 260/230 nm may originate from the production of the samples or a problem from the procedure of the RNA extraction, both need to be taken into consideration. High values of 2.3-2.4 often may come from blank measuring on an unclean pedestal or using the wrong solution as a blank on the Nanodrop. The blank used in this study was the RNAse free water and are of identical pH and ionic strength (Matlock, 2015).

The ratio of absorbance at 260 nm and 230 nm is more sensitive in detecting contaminants at 230 nm than the A260/A280. More pollutions are absorbing at 230 nm, like GTC guanidine thiocyanate, GuHCl guanidine hydrochloride, Trition X-100, Tween-20, EDTA, phenol, and proteins. Also, polysaccharides and free-floating silica fibers can be absorbed at 230nm, but not as strong an effect. Impurities of protein mainly disturb the second ratio, and therefore A260/A230 is more appropriate for measuring protein contamination. Trixtion X-100 a non-ionic detergent has an equal effect on both ratios (Koetsier and Cantor, 2019).

Particularly in plant tissue samples RNA extractions that are not entirely pure may detect high phenol signals from the remaining polyphenols. Weak signals can come from polysaccharides. These approximately only disturb the A260/A260 and causes background dispersal. Contamination from polyphenols and polysaccharides can be extracted with specific lysis chemicals (Koetsier and Cantor, 2019).

3.5 Real Time -PCR

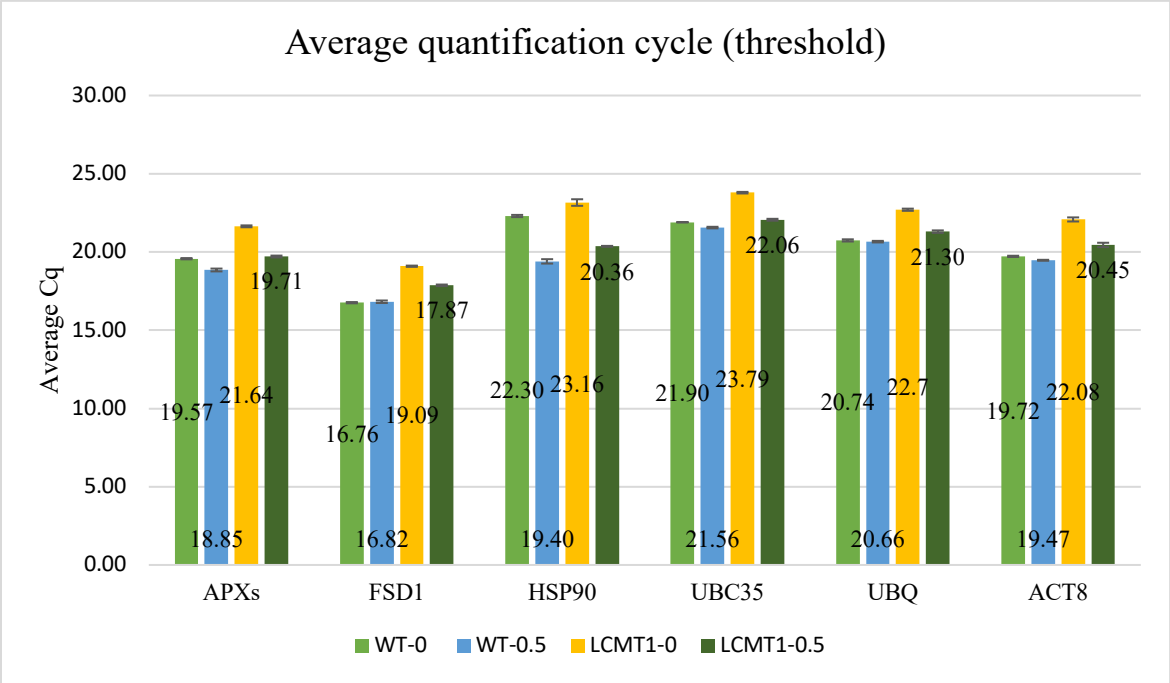

Figure 3.5.1: The mean of the quantification cycle (threshold) of the genes studies *APXs* (At4g08390), *FSD1* (At4g25100), and *HSP90* (At5g52640) that are expressed in *Arabidopsis thaliana WT* and *lcmt1* following growth on 0 mM H_2O_2 and 0.5 mM H_2O_2. Eight parallels containing five *WT* and five *lcmt1* of the same plant seed sample for both the control media ½ MS (0 mM H_2O_2) and the ½ MS with 0.5 mM H_2O_2 were used for this RT-PCR. *UBC35, UBQ,* and *ACT8* are reference genes and were used as reference internal control. The standard error is presented in the vertical bars.

The reference genes *UBQ, UBC35*, and *ACT8* were used as an internal control. Multiple reference genes for internal control are necessary, as studies have found the expression stability of the housekeeping genes not be constant over all stress conditions as earlier proposed (Regier and Frey, 2010). They were used as an endogenous control to measure the relative quantification of *APXs*, *FSD1*, and *HSP90-1*. Figure 3.5.1 signifies that *ACT8* had the lowest Cq mean in all samples, which means that the fluorescent signal of WT-0.5 (Cq mean=19.47) crossed the threshold line before any of the other reference control samples. *lcmt1* in both untreated (Cq mean=22.08) and stress treatment (20.45) had higher Cq values than WT, this may indicate that WT had a larger quantity of *ACT8* expressed.

The figure 3.4.1 shows the average quantification cycle, the Cq values were all lower than 29, which is a strong positive reaction, and indicates that the target nucleic acid in the sample is plentiful. Ct equivalent as Cq is expressed in the number of cycles that the fluorescent signal

uses to cross the threshold. When fluorescent signals are accumulated, the RT-PCR detects it as a strong positive reaction. Lower values of Cq (Ct) show the contrary values of abundant target nucleic acid, so the lower values of Cq have a higher amount of target nucleic acid present in the sample (WVDL, 2018).

lcmt1-0 had higher Cq values, the gene expression was low in all genes when compared to WT, as a result of which the fluorescent signal crossed the threshold line after the two WT samples. WT indicates to have a higher quantity of *APXs* after the H_2O_2 induced stress (Cq mean=18.85) than in the control WT-0 (Cq mean=19.57). The mutant *lcmt1*-0,5 also had a larger amount of *APXs* gene (Cq mean=19.71) than the control *lcmt1*-0 (Cq mean=21.64).

The highest expression of the *FSD1* gene was in WT-0 (Cq mean=16.76). Second, comes WT-0.5 (Cq mean=16.82), and third the stressed induced *lcmt1*-0.5 (Cq mean=17.87). *lcmt1-0* had the lowest expression of *FSD1* (Cq mean=19.09), however, this is still a strong positive reaction and there is a large amount of *FSD1* present. The plant samples from the stress-induced environment showed the greatest gene expression of *HSP90-1* WT-0.5 (Cq mean=19.40) and *lcmt1*-0.5. The gene expression of *FSD1* in WT-0 (Cq mean=22.30) and *lcmt1*-0 (Cq mean=23.16) was also at high levels.

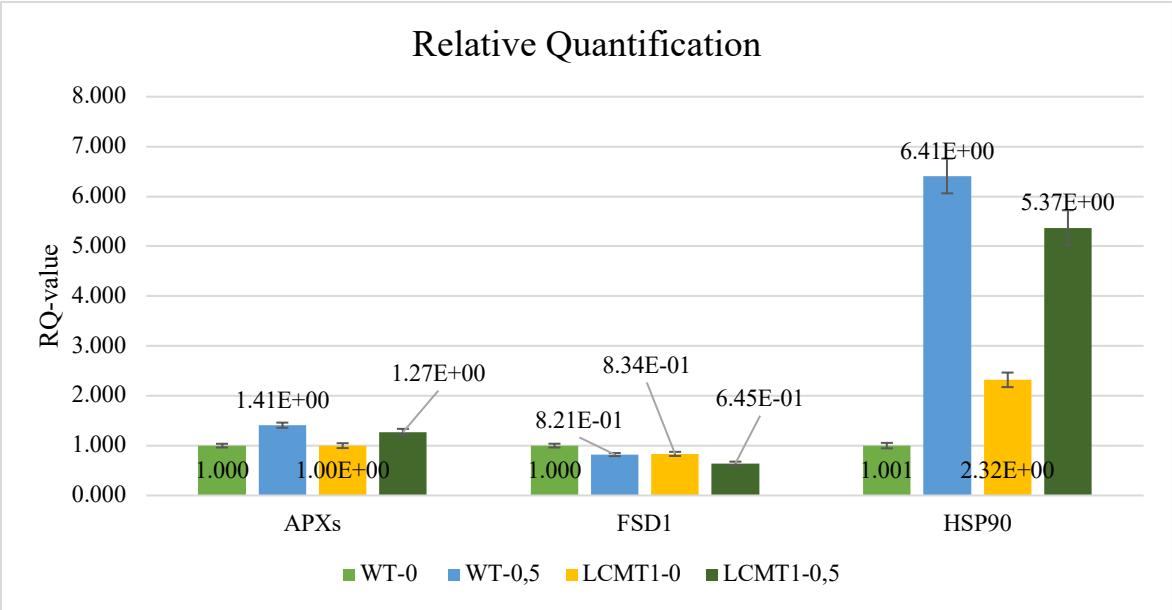

Figure 3.5.2: The mean of relative quantification of genes *APXs, FSD1, and HSP90* that are expressed in *A. thaliana* WT and *lcmt1*. *WT*-0, which is WT on regular ½ MS was used as the calibrator. RQ value equals $2^{-\Delta\Delta Ct(Cq)}$ (University of Montreal, n.d.). SE is shown in the vertical bars.

The calibrator WT-0 should have a value of 1 and our calibrator meet this requirement. RQ values need a minimum of a two-fold change to be even reviewed as significant (University of Montreal, n.d.). Relative quantification is often used to compare the variance in gene expression in two different treatments, here 0 mM H_2O_2 and 0.5 mM H_2O_2 where the levels of gene expression are normalized to the internal reference gene. WT-0 is the calibrator sample of the untreated sample, which means the expression of the target RNA in WT-0.5, l*cmt*1-0, and *lcmt1*-0.5 was relative to the identical sequence found in WT-0. By using relative quantification, we eliminate the limits of absolute quantification, where finding an appropriate internal control that is at constant levels in all samples is essential (Brunner, *et al* 2004). On the other hand, having two or more endogenous controls the results will be more precise (University of Montreal, n.d)

The fold of WT-0.5(1.4E+00) is not considered significant of the *APXs* and is a small fold. The H_2O_2 induced *lcmt1-0.5* had an RQ value (1.27E+00) and is also a small fold and not seen as noteworthy. Likewise, *lcmt1-0* had an RQ value of (1.00E+00) in the *APXs* gene. *FSD1* had contrarily RQ values than *APXs*, here the plant tissue samples were had a lower gene expression of *FSD1* than the calibrator. RQ value of WT-0.5 (8.21E-01), which means that the gene is 82 times less expressed than in WT-0. lcmt1-0.5 had an RQ of (6.45E-01) which correlates to the expression of *FSD1* to be 65 times to a lesser extent expressed here than in the calibrator. *lcmt1*-0 RQ value (8.34E-01), 83 times less expression of *FSD1* compared to WT-0.

In *HSP90-1* the gene expression showed significant fold change. WT-0.5 had the largest fold with an RQ value (6.41E+00), the *HSP90* gene was 6.4-fold higher here than in WT-0. *lcmt1*-0.5 comes second with (Rq=5.37E+00). *lcmt1* in stressed condition had 5.37-fold more *HSP90* expressed. Nonetheless *lcmt1-0* untreated only had a 2.32 higher fold.

4. Discussion

4.1 Induced chlorosis and anthocyanins by nitrogen deficiency

Observations of WT and *lcmt1* in nitrogen deficient media revealed that the low nitrogen affected the plant weight, but not necessarily the root growth. The plants that were missing both NO_3 and NH_4^+ had lower plant weight than the ones that had at least one nitrogen source in the media. Media with deficiency of both NO_3^- and NH_4^+ showed that *lcmt1* was more affected than WT when it came to plant weight. The branching also seems to have decreased in *lcmt1* compared to WT. Compared to the other media the leaves were smaller in both WT and *lcmt1* for the one with less nitrogen. The colour of the leaves was also paler, and this could be because of chlorosis, which earlier has been suggested that chlorosis could give the plant more paled or yellowish leaves (Forchhammer and Schwarz, 2018). Some leaves of both WT and *lcmt1* had a purple color and this could indicate anthocyanin production. In WT there was more anthocyanin production compared to *lcmt1*, but because of the smaller leaves in *lcmt1,* the mass was barely half of WT`s mass in this experiment. Ideally, both plant tissues should have been 0.05 g to get the best result, but in this experiment, the relative concentration of anthocyanins in WT was 4 times larger than in *lcmt1*. This could indicate higher levels of anthocyanins in WT than *lcmt1* when induced in stress conditions, but this needs further research.

4.2 Inhibited root elongation by phosphorus deficiency

The root length was most affected by the plants grown in media without P. Plants respond to less phosphorus by root apical meristem (RAM) exhaustion and the growth of the primary root reduces (Gutièrres-Alanís *et al.,* 2018; Bang et al., 2020). This could describe why the root length was shorter than the ones in other media. Some of the leaves turned out pale/yellowish which could indicate chlorosis. Also, some turned purple which could indicate anthocyanin production, but the accumulation of anthocyanin was not as high here as in the N-deficiency media.

4.3 Effects of methylation caused by nitrogen and phosphorus deficiency

In mutant *lcmt1,* only the unmethylated form of PP2A-C is present and this can affect the plants under stressful conditions (Creighton *et al.,* 2017). From the experiment with N and P deficiency, it could be that *lcmt1* was more affected by the stress factors compared to WT. The *lcmt1* in media without P had shorter roots and the leaves in the media without N had a

paler color and were less branched compared to WT, but there was not enough evidence to prove that there was more chlorosis in *lcmt1* than WT because the leaves in WT also had some pale leaves.

4.4 Effects of limitation of potassium and nitrate deficiency

In the Petri dishes with a limitation of K the growth seems not to be that affected and the leaves are also almost as big and green as the one with all the nutrients. Some of the *lcmt1* leaves turned out purple and that could indicate the production of anthocyanins, but there was not enough evidence to tell this for sure. The limitation of K needs further research.

4.5 Oxidative stress

The added 0.5 mM H_2O_2 induced oxidative stress in the seedlings of *A. thaliana* lowered both the fresh weight of WT and the knockdown *lcmt1* in comparison to the seedlings grown on the ½ MS control germination media. A study by Zhu *et al.,* 2007 supports this theory. Results from the three parallels fluctuated. Yet the knockdown *lcmt1* appeared to be the most inhibited from the treatment with H_2O_2.WT had more elongated roots and shoots, the leaves were greater in size and greener than *lcmt1* in the presence of 0.5 mM H_2O_2. However, the mutant *lcmt1* also had a lower weight and root development than WT in the control medium. Stanevich *et al.,*2011 showed in their research that *lcmt1* has reduced configuration of the holoenzyme and uncontrolled phosphatase activity, lacking these it is expected for the mutant PP2A-C methylation to be not controlled. Methylation of the C-subunit of PP2A appears to have an important role in stress environments.

In this study, the first parallel showed in figure 7.2.1 was done with 2.5 mM and 3.75 mM H_2O_2. Neither of the roots was elongated and shoots were bleached and lacked chlorophyll. The concentration was then lowered to 1.5 mM and 1 mM, here the roots did not significantly extend, but the shoots were not entirely bleached just looked more yellow and smaller, than in the control. A study from Claeys *et al*., 2014 revealed that there are more observable symptoms of stress as anthocyanins or bleaching accumulation of the shoots at concentrations of 1 mM of H_2O_2 and up. On the other hand, the first parallel had unusual morphological responses on the control media too, with no root growth and bleaching of the leaves. The mutant *lcmt1* and WT appeared to react to the induced oxidative stress in the same manner. This may indicate that the media made were not of good quality, or that the 16-hour light/8-

hour dark cycle was not followed. Therefore, the result of the first parallel not quite trustworthy.

Claeys et al., 2014 study implied that bleached plants had a significant decrease in growth over time, which can be because of the non-existent photosynthesis from the lack of chlorophyll. By observing figure 7.2.1 we are not able to specify that either one phenotype had more or less bleaching than the other. More research and parallels of this experiment of induced H_2O_2 oxidative stress on *A. thaliana* and how it affects WT and *lcmt1* need to be done to conclude which of the phenotypes response best to an increased concentration of ROS.

4.6 RNA concentration

The plant tissue sample was checked for pureness using the Nanodrop 2000. RNA isolated by using the RNAeasy kit cleanness can be calculated by finding the ratio of absorbance at A260/A280 and A260/A230. At this absorbance, the ratio will give an estimate of the purity of the RNA in plant tissue samples. Contamination such as proteins can be absorbed in this range (Qiagen, 2021).

Results from the first ratio measured of 260nm and 280nm are considered pure and are of good quality. The second ratio 260/230 nm evaluates the purity in the RNA and is extra sensitive for impurities that absorb at 230nm. Absorbing at 230nm we have GTC (guanidine thiocyanate) and GuHCl (guanidine hydrochloride), EDTA and Trition X-1000 and Tween-20. Further, also proteins and phenol can be detected at 230nm. Especially in plant samples, the RNA extraction that is impure can reveal a signal of strong phenol from polyphenols. Weak signals can also come from polysaccharides, these signals affect the A260/A230nm ratio. Contaminants absorbing at 230nm are plentiful even more than those at 280nm (Koetsier and Cantor, 2019). The estimate of the second ratio tends to be higher than in the first ratio (Scientific, 2010). Results from the second ratio showed higher values and were exceeding the acceptable limit, however, a lower value shows contamination a higher value can be background scattering.

4.7 Analysis of the gene expression

The result of the average quantification cycle showed that of the three reference genes that were used, the two highest expressed genes were *ACT8* and *UBQ*. Both would fit as a single internal control. *UBC35* was also highly expressed and would be compatible as a single endogenous control gene. In later years, it has been found that all endogenous control genes are not expressed persistently in all stress environments, that were earlier proposed (Regier and Frey, 2010). Brunner *et al.,*2014 showed in their study that this needs to be taken into consideration, especially when plant tissues are grown under different environmental stress conditions. Using two or three endogenous controls will improve the accuracy of the results.

Ascorbate peroxidase chloroplastic stromal (*APXs*) was upregulated in H_2O_2 treatment, which induced oxidative stress in *A. thaliana*. In this research, the *APXs* gene showed different expression levels in all four plant tissue samples shown in figure 3.5.1 the *APXs* gene plays a significant role in removing hydrogen peroxide at high concentrations in plant cells. All four plant tissue samples obtained a large amount of *APXs*. The highest expression of *APXs* was found in WT-0.5, this was expected as *APXs* role is to remove toxic excess of H_2O_2. However, WT-0 without any treatment had greater expression of *APXs* than *lcmt1* in both treatments. *lcmt1*-0 had the lowest expression, *lcmt1* with H_2O_2 induced stress showed elevated expression levels of *APXs*. The mutant *lcmt1* showed a higher upregulation of *APXs* from the mutant in the control medium, in comparison to WT. It appears that H_2O_2 treatment affected *lcmt1* more than its counterpart WT, and that the ROS accumulation was higher in the mutant. This indicates the importance of PP2A methylation in *A. thaliana* during oxidative stress.

Still, the most expressed gene of all was *FSD1,* this gene is (SOD) superoxide dismutase 1 and is involved in the removal of superoxide radicals an element of ROS. WT-0 had the greatest expression of *FSD1* of all four plant tissue samples. *FSD1* was downregulated in WT under a stressed environment. In the knockdown *lcmt1*, *FSD1* was upregulated. This may indicate that ROS production was higher in the *lcmt1*. Nonetheless, WT had a higher expression of the gene than the mutant. As mentioned earlier, it seemed like *lcmt1* reacts more to the hydrogen peroxide treatment.

In a study by Jacob *et al*.,2017 the gene *HSP90-1* is believed to not only be involved in response to heat stress but enrolling in numerous responses to stress conditions in plant cells.

HSP90-1 was upregulated during stressed environments in both WT and *lcmt1*. This gene had the most significant fold change. WT was 6.4-fold higher under stressed conditions. The mutant *lcmt1* had a 5.37-fold higher upon induced oxidative stress. Given that the *HSP90* plays a key role in responses to stress in plant cells, the gene expression was predicted to be high in these plant tissue samples.

LCMT1 in the mutated form is unable to methylate the C-subunit of PP2A, the methylation of PP2A-C is vital for overall cell health and biogenesis, also for the assembly of the holoenzymes of PP2A. Knockout *lcmt1* is expected to have a lower tolerance to abiotic and biotic stress conditions. In the experiment of nutrient deficiency, the methylation of PP2A-C seemed to be significant to tolerate the lack of nutrients. WT on average had longer roots, weighted more, and the growth was not impaired.

The result of the RT-PCR showed a correlation to this theory, *lcmt1* under the induced oxidative stress environment had a more noteworthy difference in terms of the cycle threshold of gene expression to its control *lcmt1-0* than WT. This means that the *lcmt*1-0.5 fluorescent signal crossed the threshold line quite before the control of the mutant. However, observing the relative quantification (figure 3.5.2) we can see that PP2A-C methylation has an impact on responses to stress. Abiotic stress such as induced levels of H_2O_2 amplifies the rate of ROS production. Eukaryotes have mechanisms that detoxify ROS, such as the genes *APXs* and *FSD1* are decontaminating enzymes in plants (Zhu *et al*.,2007). In our study, WT appeared to be more tolerant to direct oxidative stress than *lcmt1*. This shows the importance of properly oxidative protection. WT-0.5 in the stressed environment had higher levels of *FSD1, APXs,* and *HSP90-1* in contrast to the knockdown *lcmt1* in the same treatment of 0.5 mM H_2O_2. The role of methylation of PP2A in *A. thaliana* signifies to have a huge impact during the induced oxidative stress. It seemed like WT had more protection from oxidative stress, and the LCMT1 enzyme gave the impression of being important in this stress condition. *lcmt1* has an unnormal function of the LCMT1 enzyme and therefore will have no control of the phosphatase activity and no activation of the holoenzymes. Possibilities of this knockout to be harmful to cell survival, biosynthesis and normal functions in the cell are very probable (Stanevich *et al*.,2011).

4.8 Sources of error

When doing the measurement of anthocyanin, the concentration of WT was four times larger than in *lcmt1*. This was because *lcmt1* was more affected by the low nitrogen and regardless of many parallels, the plant weight didn`t become more than 25 mg. This affected the anthocyanin measurement because the concentration of WT (50 mg) was double as much as *lcmt1*. The first experiment with different nutrients was done three times, and some grew for 6 days and some for 7 days with a 16-h light/8-h dark cycle. Some Petri dishes were placed longer in the fridge before taken to the 16-h light/8-h dark cycle room as well. This could affect the growth and the weight of the plants in all of the Petri dishes. This is also relevant for the experiment with H_2O_2 where this also was placed for different days in a 16-h light/8-h dark cycle. The lights were also turned off where the Petri dishes were placed for some time, and this can also affect the growth of the plants. Contamination was also a problem, with many Petri dishes that had mould and bacteria inside. There was also a problem with old seeds that didn't grow so the experiment had to be done many times. The experiments with higher H_2O_2 concentration were not successful, this could be because the H_2O_2 was really effective and totally inhibited the plant development. The gene expression analysis was only performed once, and therefore the result cannot be fully trusted.

5. Conclusion and future perspective

5.1 Conclusion

This study aimed to investigate and reveal how significant the methylation of PP2A-C and the LCMT1 gene, which is the regulator for this methylation is for cell survival and health. The research was done by comparing the knockout *lcmt1* to WT under various stressed environments. Our study revealed:

- In germination media ½ MS with 1% sucrose, the weight of the mutant *lcmt1* was significantly lower compared to WT. The leaves of WT appeared longer and greener.
- The deficiency of nitrogen affected both WT and *lcmt1,* the shoots indicated both anthocyanin production and chlorosis.
- The deficiency of phosphorus affected the root elongation the most and some purple leaves indicated some anthocyanin production.
- The deficiency of potassium and nitrate showed less inhibition on the roots of *lcmt1*, the plant weight did not get affected on either WT or *lcmt1*. The leaves turned out as green, but the shoots were not as elongated as in the control media. In one of the parallels the leaves of *lcmt1* indicated anthocyanin production.
- The knockout *lcmt1* was more inhibited from H_2O_2 induced oxidative stress, the root growth was reduced, and the shoots were smaller than WT.
- In high concentrations of H_2O_2, the shoots of WT and *lcmt1* were bleached and lacked chlorophyll, and the plants had no noteworthy root elongation.
- *APXs, FSD1* and *HSP90* genes were expressed at higher levels in WT in comparison to *lcmt1*, in both control and stressed treatment.
- The ROS scavenge genes *APXs* and *FSD1* were upregulated at higher levels in the knockdown *lcmt1*, which may indicate that the mutant´s ROS production was higher.

5.2 Future perspective

Some factors have shown that the methylation of PP2A could be important when plants need to adapt to different nutrient deficiencies. Adaption to nitrogen and phosphorus deficiency is shown to be important and continuing with these experiments could be interesting. This would involve experimentation with more parallels, and plants grown on rockwool for several weeks. It also would be interesting to see how chlorosis and anthocyanins production affected the plant growth in nitrogen deficiency media and compare the difference in anthocyanin production between WT and the knockout *lcmt1*. Further, investigate more how the limitation of potassium (K) affects *A. thaliana* or other plants, and grow the plants on rockwool here as well. Additionally, do more research on the gene expression in the knockout *lcmt1* during oxidative stress, as the ROS production seemed to be higher in the mutant. The effects of oxidative stress could also be experimented further by having more parallels than one to compare the gene expressions. Genes that are involved with the adaptation to nutrient limitation could be of interest as well, where a RT-PCR test also could be done for the experiments with deficiency of N, P, and K.

6. Reference list:

Anon, 2020. *chlorosis*. Encyclopædia Britannica Online. From https://bibsys-almaprimo.hosted.exlibrisgroup.com/permalink/f/35bh5q/TN_cdi_britannica_eb_82261

Applied biosystems. 2016. *Real-Time PCR: understanding Ct*. Thermo Fisher Scientific Inc.

Applied biosystems. n.d. *How TaqMan assays work*. Retrieved from Thermo Fisher https://www.thermofisher.com/no/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center/real-time-pcr-basics/how-taqman-assays-work.html

Applied biosystems. 2010. *Taqman® Universal Master Mix II*, Thermo Fisher Scientific Inc.

Applied biosystems, 2009. NanoDrop 2000/2000c Spectrophotometer User Manual. Thermo Fisher Scientifc Inc*, Delaware, USA*.

Applied biosystems., 2010. *T042-Technical Bulletin NanoDrop Spectrophotometers, 260/280 and 260/230 Ratios.* , Thermo Fisher Scientific Inc.

Asad, S.A., Muhammad, S., Farooq, M., Afzal, A., Broadley, M., Young, S. and West, H., 2015. Anthocyanin production in the hyperaccumulator plant Noccaea caerulescens in response to herbivory and zinc stress. *Acta physiologiae plantarum*, *37*(1), pp.1-9.https://doi.org/10.1007/s11738-014-1715-5

Bang, T.C et al., 2021. The molecular–physiological functions of mineral macronutrients and their consequences for deficiency symptoms in plants. *The New phytologist*, 229(5), pp.2446–2469 https://doi.org/10.1111/nph.17074

Bharadwaj, S., Ali, A. and Ovsenek, N., 1999. Multiple components of the HSP90 chaperone complex function in regulation of heat shock factor 1 in vivo. *Molecular and cellular biology*, *19*(12), pp.8033-8041.https://doi.org/10.1128/mcb.19.12.8033

Brunner, A.M., Yakovlev, I.A. and Strauss, S.H., 2004. Validating internal controls for quantitative plant gene expression studies. *BMC plant biology*, *4*(1), pp.1-7. https://doi.org/10.1186/1471-2229-4-14

Campbell, N.A. et al., 2018. *Biology: a global approach 11th ed*., global., New York: Pearson Education, pp 1298-1300

Claeys, H., Van Landeghem, S., Dubois, M., Maleux, K. and Inzé, D., 2014. What is stress? Dose-response effects in commonly used in vitro stress assays. *Plant physiology*, *165*(2), pp.519-527. https://doi.org/10.1104/pp.113.234641

Creighton, M.T et al., 2017. Methylation of protein phosphatase 2A—Influence of regulators and environmental stress factors. *Plant, cell and environment*, 40(10), pp.2347–2358. https://doi.org/10.1111/pce.13038

Creighton, M.T. & Universitetet i Stavanger Det teknisk-naturvitenskapelige fakultet, 2017. *PP2A and PP4 in Arabidopsis: regulation and influence*, no. 361.

Dvorak, P., Krasylenko, Y., Ovecka, M., Basheer, J., Zapletalova, V., Samaj, J. and Takac, T., 2020. FSD1: a plastidial, nuclear and cytoplasmic enzyme relocalizing to the plasma membrane under salinity. *bioRxiv*. https://doi.org/10.1101/2020.03.24.005363

Forchhammer, K & S, Rakefet, 2019. Nitrogen chlorosis in unicellular cyanobacteria – a developmental program for surviving nitrogen deprivation. *Environmental microbiology*, 21(4), pp.1173–1184. https://doi.org/10.1111/1462-2920.14447

Gachon, C, Mingam, A & Charrier, B, 2004. Real-time PCR: what relevance to plant studies? *Journal of experimental botany*, 55(402), pp.1445–1454. https://doi.org/10.1093/jxb/erh181

Grierson, C., Nielsen, E., Ketelaarc, T. and Schiefelbein, J., 2014. Root hairs. *The Arabidopsis Book/American Society of Plant Biologists*, *12*.https://doi.org/10.1199/tab.0172

Guénin, Stéphanie et al., 2009. Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. *Journal of experimental botany*, 60(2), pp.487–493. https://doi.org/10.1093/jxb/ern305

Jacob, P, Hirt, Heribert & Bendahmane, Abdelhafid, 2017. The heat-shock protein/chaperone network and multiple stress resistance. *Plant biotechnology journal*, 15(4), pp.405–414. https://doi.org/10.1111/pbi.12659

Jacquot, J.P., 2009. *Oxidative stress and redox regulation in plants*. Academic Press.

Kliebenstein, D.J., Rita-Ann Monde & Last, R.L. 1998, "Superoxide dismutase in Arabidopsis: An eclectic enzyme family with disparate regulation and protein localization", *Plant Physiology,* vol. 118, no. 2, pp. 637-50.https://doi.org/10.1104/pp.118.2.637

Koetsier, G. and Cantor, E., 2019. A practical guide to analyzing nucleic acid concentration and purity with microvolume spectrophotometers. *New England Biolabs Inc*.

Koornneef, M. and Meinke, D., 2010. The development of Arabidopsis as a model plant. *The Plant Journal*, *61*(6), pp.909-921.

Kovinich, N., Kayanja, G., Chanoca, A., Otegui, M.S. and Grotewold, E., 2015. Abiotic stresses induce different localizations of anthocyanins in Arabidopsis. *Plant signaling & behavior*, *10*(7), p.e1027850. https://doi.org/10.1080/15592324.2015.1027850

Lea, P. J., & Morot-Gaudry, J.-F. (2001). *Plant nitrogen* (p 1, pp. 61-71). Springer.

Lemaitre, T. et al., 2008. Enzymatic and metabolic diagnostic of nitrogen deficiency in Arabidopsis thaliana Wassileskija accession. *Plant and cell physiology*, 49(7), pp.1056–1065. https://doi.org/10.1093/pcp/pcn081

Life science (2018), *Lightcycler® 96 instrument*, Roche Molecular Systems, Inc

Lillo, C., Kataya, A.R., Heidari, B., Creighton, M.T., NEMIE-FEYISSA, D.U.G.A.S.S.A., Ginbot, Z. and Jonassen, E.M., 2014. Protein phosphatases PP 2A, PP 4 and PP 6: mediators and regulators in development and responses to environmental cues. *Plant, cell & environment*, *37*(12), (pp.2631-2648)https://doi.org/10.1111/pce.12364

Logan, J., Edwards, K. J., & Saunders, N. A. (2009). Real-time PCR: current technology and applications, in P Bustin, S.A, & Noland, T (eds), *Analysis of mRNA Expression by Real-time PCR,* Caister Academic Press. (pp. 111-115).

Logan, J., Edwards, K. J., & Saunders, N. A. (2009). *Real-time PCR: current technology and applications*, in P Logan. J & Edwards, K. (eds), *An Overview of PCR Platforms,* Caister Academic Press. (p. 8).

Logan, J., Edwards, K. J., & Saunders, N. A. (2009*).* Real-time PCR: current technology and applications, in P Vandesompele, J.,Kubista, M & Pfaffl, M (eds), *Reference Gene Validation Software for Improved Normalization*, Caister Academic Press. (p. 47).

Mager, S & Ludewig, U, 2018. Massive Loss of DNA Methylation in Nitrogen-, but Not in Phosphorus-Deficient Zea mays Roots Is Poorly Correlated with Gene Expression Differences. *Frontiers in plant science*, 9, p.497. https://doi.org/10.3389/fpls.2018.00497

Massaro, M, De Paoli, E, Thomasi, N, Morgante, M, Pinton R & Zanin, L., 2019. Transgenerational Response to Nitrogen Deprivation in Arabidopsis thaliana*. International journal of molecular sciences,* 20(22), p.5587.https://doi.org/10.3390/ijms20225587

Matlock, B. (2015). *Assessment of Nucleic Acid Purity*. Wilmington, MA, USA: Thermo Fisher Scientific.

McPherson, M. J., & Møller, S. G. (2006). *PCR* (2nd ed., pp. 209-215). Taylor & Francis.

Meyerowitz, E.M. & Somerville, C.R., 1994. *Arabidopsis*, New York: Cold Spring Harbor Lab. Press.

Poirier, Y & Bucher, M, 2002. Phosphate transport and homeostasis in Arabidopsis. *The Arabidopsis book,* 1, p.e0024. https://dx.doi.org/10.1199%2Ftab.0024

Regier, N. and Frey, B., 2010. Experimental comparison of relative RT-qPCR quantification approaches for gene expression studies in poplar. *BMC molecular biology*, *11*(1), pp.1-8.https://doi.org/10.1186/1471-2199-11-57

Stanevich, V., Jiang, L., Satyshur, K.A., Li, Y., Jeffrey, P.D., Li, Z., Menden, P., Semmelhack, M.F. and Xing, Y., 2011. The structural basis for tight control of PP2A methylation and function by LCMT-1. *Molecular cell*, *41*(3), pp.331-342. https://doi.org/10.1016/j.molcel.2010.12.030

Stephenson, F.H., 2016. *Calculations for molecular biology and biotechnology Third.,* Amsterdam, Netherlands: Academic Press. In chapter 9 – *Real Time PCR* (pp.215-320)

Sultana, R., Nakayama, K., Nakamura, K. and Kyo, S., 2016. PPP2R1A (protein phosphatase 2 regulatory subunit A, alpha). *Atlas of Genetics and Cytogenetics in Oncology and Haematology*. https://doi.org/10.4267/2042/66936

Sun, Ji & Zheng, Ning, 2015. Molecular Mechanism Underlying the Plant NRT1.1 Dual-Affinity Nitrate Transporte*r*. *Frontiers in physiology*, 6, p.386. https://dx.doi.org/10.3389%2Ffphys.2015.00386

Sustr, M, Soukup, A & Tylova, E, 2019. Potassium in Root Growth and Development*.* *Plants (Basel),* 8(10), p.435. https://dx.doi.org/10.3390%2Fplants8100435

TAIR. (2013) *Arabidopsis.org*. Retrieved from Locus: AT4G25100: https://www.arabidopsis.org/servlets/TairObject?id=126924&type=locus

TAIR (2013) *Arabidopsis.org*. Retrieved from Locus AT1G1G49240: https://www.arabidopsis.org/servlets/TairObject?id=29656&type=locus

TAIR. (2015). *Arabidopsis.org*. Retrieved from Locus: AT1g78870:https://www.arabidopsis.org/servlets/TairObject?id=31115&type=locus

TAIR (2015) *Arabidopsis.org*. Retrieved from Locus: AT5G52640: https://www.arabidopsis.org/servlets/TairObject?id=131312&type=locus

TAIR (2015) *Arabidopsis.org*. Retrieved from Locus: AT4G05320: https://www.arabidopsis.org/servlets/TairObject?id=129794&type=locus

TAIR (2020) *Arabidopsis.org*. Retrieved from Locus: AT4G05320:https://www.arabidopsis.org/servlets/TairObject?id=126703&type=locus

University of Montreal, Institute of research in immunology and cancer. Genomic Platform. Information of qPCR results n.d., viewed 02 June 202: https://genomique.iric.ca/resources/files/How_to_deal_with_qPCR_results.pdf

Wisconsin Veterinary Diagnostic Laboratory (WVDL), 2018. *What does CT mean?* https://www.wvdl.wisc.edu/wp-content/uploads/2018/05/What-does-CTmeanfinahandoutlJanuary2014.pdf

Wu, B. and Wang, B., 2019. Comparative analysis of ascorbate peroxidases (APXs) from selected plants with a special focus on Oryza sativa employing public databases. *PloS one*, *14*(12), p.e0226543 https://doi.org/10.1371/journal.pone.0226543

Zhu, J., Fu, X., Koo, Y.D., Zhu, J.K., Jenney Jr, F.E., Adams, M.W., Zhu, Y., Shi, H., Yun, D.J., Hasegawa, P.M. and Bressan, R.A., 2007. An enhancer mutant of Arabidopsis salt overly sensitive 3 mediates both ion homeostasis and the oxidative stress response. *Molecular and cellular biology*, *27*(14), pp.5214-5224.https://doi.org/10.1128/MCB.01989-06

7. Appendix

7.1 Experiment 1, WT and lcmt1 growth on different kinds of stress media, and one control media.

Table 7.1.1 The average plant weight in the three parallels of experiment with ½ MS, w/o KNO_3 and NH_4NO_3, w/o KH_2PO_4 and KH_2PO_4 switched with NaH_2PO_4 and w/o KNO_3.

½ MS	WT (g)	lcmt1 (g)
Parallel 1	0.075	0.032
Parallel 2	0.040	0.022
Parallel 3	0.056	0.050
Mean	0.057	0.035
SD	0.020	0.016
SE	0.007	0.005
p-value	0.020	
Without KNO_3 and NH_4NO3	WT (g)	lcmt1 (g)
Parallel 1	0.013	0.012
Parallel 2	0.035	0.021
Parallel 3	0.020	0.018
Mean	0.023	0.017
SD	0.015	0.005
SE	0.005	0.002
p-value	0.276	
Without KH_2PO_4	WT (g)	lcmt1 (g)
Parallel 1	0.031	0.017
Parallel 2	0.036	0.039
Parallel 3	0.052	0.047
Mean	0.040	0.034
SD	0.012	0.014
SE	0.004	0.005
p-value	0.388	
With NaH_2PO_4, without KNO_3	WT (g)	lcmt1 (g)
Parallel 1	0.042	0.039
Parallel 2	0.069	0.066
Parallel 3	0.052	0.057
Mean	0.054	0.054
SD	0.030	0.017
SE	0.010	0.006
p-value	0.974	

Table 7.1.2: The root growth (mm) of *A. thaliana* on Petri dishes, the first parallel of the experiment 1.

	½ MS 1% Sucrose	
	WT (mm)	lcmt1 (mm)
	54.276	23.048
Parallel 1	60.718	33.982
	83.858	11.833
	82.431	58.112
	85.091	60.989
Parallel 2	7.5780	35.163
	29.712	31.902
	80.561	32.981
	76.077	37.295
	24.615	21.555
Mean	58.492	34.686
SD	28.502	15.235
SE	9.013	4.817
p-value	0.035	

	½ MS 1% Sucrose w/o KNO_3 and NH_4NO_3	
	WT (mm)	lcmt1 (mm)
Parallel 1	48.142	28.234
	76.971	91.964
	75.916	77.721
	61.592	50.367
	74.843	45.108
Parallel 2	61.386	85.507
	53.993	85.863
	79.413	90.079
	85.976	57.032
	44.497	83.494

Parallel 3	72.509	85.136
	66.045	92.210
	50.988	29.833
	45.662	101.926
	43.005	33.703
Parallel 4	71.178	59.728
	72.142	56.378
	72.813	37.989
	64.882	40.593
	60.365	32.817
Mean	64.116	63.284
SD	12.825	24.995
SE	2.868	5.589
p-value	0.896	
	½ MS 1% Sucrose w/o KH_2PO_4	
	WT (mm)	lcmt1 (mm)
Parallel 1	56.350	14.515
	51.539	47.983
	29.627	17.100
	41.760	5.910
	28.473	38.264
Parallel 2	47.228	42.610
	49.687	43.379
	55.466	33.386
	53.317	30.861
	56.895	54.476
Parallel 3	26.431	45.031
	13.150	26.703
	25.125	48.383
	15.517	32.139
	34.927	54.903
Parallel 4	39.763	56.514

	27.988	28.511
	60.688	52.835
	30.611	37.196
	57.337	39.580
Mean	40.094	37.514
SD	14.876	14.082
SE	3.326	3.149
p-value	0.577	

	½ MS 1% Sucrose with $Na_2H_2PO_4$, w/o KNO_3	
	WT (mm)	lcmt1 (mm)
Parallel 1	68.138	20.300
	62.147	65.737
	68.589	71.807
	65.871	40.221
	63.641	45.101
Parallel 2	23.910	104.52
	74.969	72.136
	16.206	93.500
	16.669	101.67
	32.428	72.627
Mean	49.257	68.762
SD	23.852	27.368
SE	7.543	8.655
p-value	0.107	

Table 7.1.3. The second parallel of experiment 1, the root growth (mm) of *A.thaliana* in Petri dishes.

	½ MS 1% Sucrose	
	WT (mm)	lcmt1 (mm)
	96.025	75.639
Parallel 1	89.508	74.262

	73.718	35.363
	35.951	74.732
	57.945	39.059
Parallel 2	86.369	65.621
	65.466	63.937
	65.913	50.407
	50.582	20.759
	37.162	37.939
Parallel 3	96.607	64.256
	28.761	12.847
	92.908	55.480
	26.981	74.225
	94.657	75.869
Mean	66.570	54.693
SD	25.846	20.993
SE	6.673	5.420
p-value	0.178	

	½ MS 1% Sucrose w/o KNO_3 and NH_4NO_3	
	WT (mm)	lcmt1 (mm)
	76.314	44.072
Parallel 1	65.235	24.620
	66.982	33.100
	77.160	81.677
	74.321	20.481
Parallel 2	64.324	59.813
	60.524	19.241
	65.002	36.256
	66.245	38.840

	51.215	15.269
Parallel 3	67.261	33.444
	67.662	69.141
	67.906	74.624
	66.643	78.436
	58.440	80.526
Mean	66.349	47.303
SD	6.643	24.319
SE	1.715	6.279
p-value	0.001	

	½ MS 1% Sucrose without KH_2PO_4	
	WT (mm)	lcmt1 (mm)
	42.882	34.015
Parallel 1	50.462	17.856
	39.812	18.548
	48.239	16.790
	31.566	48.152
Parallel 2	39.630	12.659
	34.070	7.094
	55.698	14.853
	31.337	12.279
	40.923	12.803
Parallel 3	53.926	19.371
	52.858	29.650
	33.835	22.226
	49.191	16.396
	45.759	17.375
Mean	43.346	20.005
SD	8.287	10.291
SE	2.140	2.657
p-value	2.38E-07	

	½ MS 1% Sucrose with $Na_2H_2PO_4$, w/o KNO_3	
	WT (mm)	lcmt1 (mm)
	65.015	26.602
Parallel 1	58.572	32.234
	64.126	108.97
	64.123	39.071
	80.465	48.055
Parallel 2	27.166	85.165
	27.808	30.412
	64.316	80.264
	44.329	62.601
	76.278	56.938
Parallel 3	72.439	24.801
	73.858	30.282
	35.730	24.485
	21.700	66.574
	22.020	100.363
Mean	53.196	54.454
SD	21.170	28.530
SE	5.466	7.366
p-value	0.891	

Table 7.1.4: The root growth (mm) of *A.thaliana* on Petri dishes the third parallel of the experiment 1.

	½ MS w. 1% Sucrose	
	WT (mm)	lcmt1 (mm)
Parallel 1	82.610	55.293
	63.130	68.139
	88.340	71.373
	56.737	75.840
	74.347	69.451
Parallel 2	63.150	73.848
	62.863	75.914
	61.026	60.953

	62.938	63.132
	68.405	69.909
Parallel 3	91.509	69.066
	67.050	67.349
	44.541	48.918
	80.678	96.461
	77.742	57.177
Mean	69.672	68.188
SD	12.706	11.028
SE	3.281	2.847
p-value	0.735	

	½ MS 1% Sucrose w/o KNO_3 and NH_4NO_3	
	WT (mm)	lcmt1 (mm)
Parallel 1	69.578	61.791
	66.686	63.342
	65.879	61.210
	73.905	64.345
	24.951	64.296
Parallel 2	51.132	68.417
	62.456	62.956
	63.093	64.090
	65.274	62.216
	71.953	64.334
Parallel 3	65.798	76.627
	61.384	70.652
	57.298	60.963
	76.829	70.334
	63.566	64.369
Mean	62.652	65.329
SD	12.221	4.341
SE	3.155	1.121

p-value	0.435

	½ MS 1% Sucrose w/o KH_2PO_4	
	WT (mm)	lcmt1 (mm)
Parallel 1	30.835	36.301
	43.596	39.625
	42.507	39.267
	45.722	26.757
	49.018	26.667
Parallel 2	47.162	42.540
	43.553	41.525
	42.608	36.799
	47.246	35.636
	52.086	40.114
Parallel 3	53.658	23.638
	39.475	29.922
	59.518	19.223
	55.097	34.167
	52.646	32.177
Mean	46.982	33.624
SD	7.126	7.030
SE	1.840	1.815
p-value	1.75E-05	

	½ MS 1% Sucrose w. NaH_2PO_4, w/o KNO_3	
	WT (mm)	lcmt1 (mm)
Parallel 1	87.899	72.899
	86.823	64.347
	78.985	80.029
	79.028	72.086
	76.966	73.743
Parallel 2	78.913	78.737

	86.870	77.642
	83.037	71.731
	83.620	69.430
	77.722	60.460
Parallel 3	88.679	63.616
	84.788	85.730
	74.469	63.513
	70.256	32.860
	79.374	75.557
Mean	81.162	69.492
SD	5.345	12.320
SE	1.380	3.181
p-value	0.003	

Relative Concentration of Anthocyanins Calculations:

Formula for relative concentration to find concentration of anthocyanins:

$$(1)\ Relative\ C_{anthocyanins} = \frac{(absorbance\ at\ 535nm) - (absorbance\ at\ 657nm)}{plant\ material\ (g)}$$

$$(1)\ Relative\ C(WT)_{anthocyanins} = \frac{0.620 - 0.020}{0.047g} = 12.8\ \mathrm{l/g}$$

$$(1)\ Relative\ C(lcmt1)_{anthocyanins} = \frac{0.020 - 0.0090}{0.025g} = 3.040\ \mathrm{l/g}$$

7.2 Experiment 2, *A. thaliana* WT and *lcmt1* growth under induced oxidative stress from addition of H_2O_2.

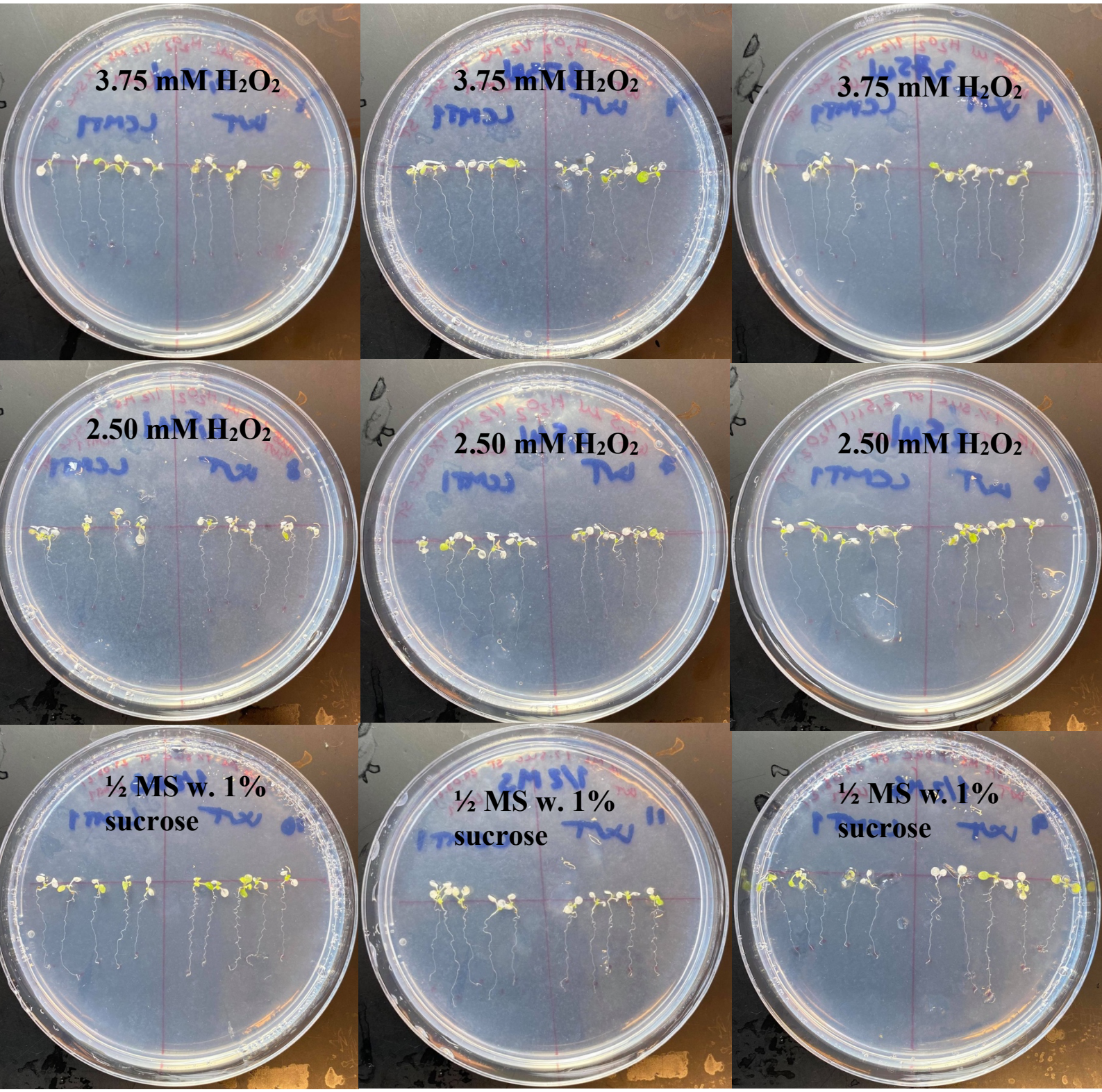

Figure 7.2.1: *Arabidopsis thaliana* phenotypes WT and *lcmt1* growth on ½ MS media with 3.75 mM and 2.5 mM H_2O_2 media and one control media of ½ MS with 1% sucrose germination media.

Table 7.2.1 The average weight of *A.thaliana* from the failed parallel of experiment 2 with 0 mM, 1 mM, 1.5mM, 2.5 mM and 3.75 mM H_2O_2.

0 mM H_2O_2	WT (g)	lcmt1 (g)
Parallel 1	0.082	0.071
Parallel 2	0.076	0.064
Parallel 3	0.063	0.059
Mean	0.079	0.072
SD	0.003	0.009
p-value	0.256	
1 mM H_2O_2	WT (g)	lcmt1 (g)
Parallel 1	0.061	0.056
Parallel 2	0.050	0.060
Parallel 3	0.035	0.025
Mean	0.048	0.047
SD	0.013	0.019
SE	0.007	0.011
p-value	0.897	
1.5 mM H_2O_2	WT(g)	lcmt1(g)
Parallel 1	0.027	0.044
Parallel 2	0.050	0.049
Parallel 3	0.030	0.029
Mean	0.035	0.040
SD	0.012	0.010
SE	0.007	0.006
p-value	0.579	
2.5 mM H_2O_2	WT(g)	lcmt1(g)
Parallel 1	0.013	0.016
Parallel 2	0.021	0.019
Parallel 3	0.019	0.017
Mean	0.018	0.017
SD	0.004	0.002
SE	0.002	0.001
p-value	0.905	
3.75 mM H_2O_2	WT(g)	lcmt1(g)
Parallel 1	0.018	0.014
Parallel 2	0.022	0.010
Parallel 3	0.013	0.013
Mean	0.018	0.012
SD	0.005	0.002
SE	0.003	0.001
p-value	0.160	

Table 7.2.2: The average weight of *A.thaliana* in the last two parallels of experiment 2 that were more successful, with concentrations of 0 mM and 0.5 mM H_2O_2.

0 mM H_2O_2	WT(g)	lcmt1(g)
Mean	0.059	0.056
SD	0.018	0.019
SE	0.004	0.004
p-value	0.520	
0.5 mM H_2O_2	WT(g)	lcmt1(g)
Mean	0.055	0.044

SD	0.014	0.012
SE	0.003	0.003
p-value	0.006	

Table 7.2.3: The root growth (mm) of *A.thaliana* on Petri dishes treated with 0.5 mM H_2O_2 second and third parallel of the experiment 2.

	½ MS 1% Sucrose w. 0.5 mM H_2O_2		½ MS 1% Sucrose w. 0.5 mM H_2O_2	
	WT (mm)	lcmt1 (mm)	WT (mm)	lcmt1 (mm)
Parallel 1	43.641	31.804	37.903	40.413
	53.038	38.027	43.617	37.853
	47.364	29.933	29.616	39.343
	37.447	30.893	57.090	38.611
	40.269	28.897	37.617	40.080
Parallel 2	52.457	49.947	21.897	14.434
	45.041	58.017	27.226	16.504
	42.711	50.520	32.286	14.146
	42.088	32.838	21.804	21.846
	37.749	34.712	24.319	16.089
Parallel 3	44.810	43.118	48.792	13.217
	60.945	49.383	19.405	13.674
	45.982	49.724	38.097	23.706
	64.435	42.003	33.565	14.632
	51.203	43.049	23.997	15.243
Parallel 4	48.282	52.326	22.188	20.045
	47.293	55.914	20.256	31.206
	44.822	66.579	33.760	28.026
	49.543	63.746	35.548	24.233
	55.111	50.886	19.056	15.901
			17.576	19.520
Parallel 5	41.676	46.066	19.996	13.204
	40.326	39.989	24.491	16.495
	42.838	40.328	31.456	16.096
	41.005	40.727	49.118	13.977
	45.770	33.075	18.462	16.368
Parallel 6	65.712	44.166	26.453	16.523

	59.443	39.717	28.551	14.615
	55.629	31.127	21.709	25.457
	49.017	35.588	30.355	28.062
	51.701	47.108	18.462	16.368
Parallel 7	43.992	17.914	33.066	17.092
	60.872	30.881	33.972	21.513
	48.180	41.002	39.960	17.305
	48.771	35.655	22.396	17.682
	33.709	45.570	30.754	18.584
Parallel 8	52.136	44.810	41.460	14.794
	26.602	41.136	42.778	17.402
	35.434	30.430	57.307	17.152
	32.584	33.516	46.850	16.924
	34.775	33.534	51.786	19.569
Mean	46.326	41.366	32.413	20.938
SD	8.837	10.115	11.020	8.215
SE	1.384	1.599	1.742	1.300
p-value	0.015		1.3E-06	

Table 7.2.4: The root growth(mm) of *A.thaliana* on control medium(1/2 MS) on Petri dishes, the second and third parallel of the experiment 2.

	½ MS 1% Sucrose		½ MS 1% Sucrose	
	WT (mm)	lcmt1 (mm)	WT (mm)	lcmt1 (mm)
Parallel 1	67.315	49.456	56.347	59.977
	63.103	40.526	57.781	65.668
	62.848	54.545	51.508	60.906
	47.540	60.868	58.880	30.664
	55.655	59.283	46.418	66.170
Parallel 2	65.563	59.971	65.690	61.464
	52.232	51.808	58.750	38.780
	49.904	62.455	55.366	44.939
	48.761	57.076	64.516	56.248
	53.560	55.347	67.242	48.824
Parallel 3	47.508	38.574	54.573	53.402

	60.404	43.484	60.260	47.363
	55.556	47.036	69.596	55.191
	53.471	47.478	64.448	45.263
	53.444	43.291	67.472	43.636
Parallel 4	59.456	63.787	66.067	26.221
	60.555	66.040	63.487	52.582
	64.280	45.945	60.295	54.071
	59.651	41.903	63.829	61.850
	53.969	38.974	69.239	43.273
Parallel 5	63.460	54.123	58.197	60.408
	59.489	60.168	61.080	54.610
	65.621	60.378	59.027	44.060
	64.612	62.076	58.738	53.346
	54.664	56.101	59.149	45.554
Parallel 6	52.667	49.332	53.166	40.703
	51.658	55.428	58.904	45.176
	53.855	44.054	64.165	72.057
	54.465	52.124	73.207	15.786
	47.599	51.026	66.649	71.149
Parallel 7	50.641	54.695	64.412	66.609
	44.475	66.000	47.587	61.800
	43.264	57.511	47.616	23.798
	50.892	44.111	59.736	59.554
	53.597	40.575	62.056	53.044
Parallel 8	50.779	53.128	57.098	53.882
	55.071	41.749	67.128	65.947
	48.207	46.885	75.531	60.578
	49.858	46.769	53.078	60.435
	65.123	40.500	75.954	55.977
Mean	55.369	51.615	61.106	52.024
SD	6.435	8.101	7.030	12.689
SE	1.017	1.281	1.112	2.006
p-value	0.025		0.0001	

7.3 RT-PCR results

Table 7.3.1: The result of the Real time-PCR done on samples four different plant tissue samples WT-0, WT-0.5, *lcmt-0* and *lcmt1-0.5.* Values Cq mean and Cq error used in calculating the average quantification cycle (figure 3.5.1). RQ mean and RQ error used in calculation of the relative quantification (figure 3.5.2).

Sample	**Gene Name**	**Cq**	**Cq Mean**	**Cq Error**	**Gene Type**	**RQ Mean**	**RQ Error**
WT-0	APXs	19.57	19.57	0.04	Target	1.000	3.7E-02
WT-0.5	APXs	18.88	18.85	0.09	Target	1.4E+00	5.2E-02
lcmt1-0	APXs	21.57	21.64	0.06	Target	1.0E+00	4.8E-02
lcmt1-0.5	APXs	19.77	19.71	0.06	Target	1.3E+00	6.6E-02
WT-0	FSD1	16.71	16.76	0.04	Target	1.000	3.8E-02
WT-0.5	FSD1	16.81	16.82	0.08	Target	8.2E-01	3.1E-02
lcmt1-0	FSD1	19.13	19.09	0.04	Target	8.3E-01	4.1E-02
lcmt1-0.5	FSD1	17.83	17.87	0.05	Target	6.5E-01	3.4E-02
WT-0	HSP90	22.29	22.30	0.07	Target	1.001	5.5E-02
WT-0.5	HSP90	19.29	19.40	0.14	Target	6.4E+00	3.5E-01
lcmt1-0	HSP90	23.41	23.16	0.21	Target	2.3E+00	1.5E-01
lcmt1-0.5	HSP90	20.34	20.36	0.03	Target	5.4E+00	3.5E-01
WT-0	UBC35	21.90	21.90	0.00	Reference		
WT-0.5	UBC35	21.57	21.56	0.05	Reference		
lcmt1-0	UBC35	23.79	23.79	0.05	Reference		
lcmt1-0.5	UBC35	22.00	22.06	0.06	Reference		
WT-0	UBQ	20.67	20.74	0.07	Reference		
WT-0.5	UBQ	20.69	20.66	0.05	Reference		
lcmt1-0	UBQ	22.78	22.70	0.07	Reference		
lcmt1-0.5	UBQ	21.24	21.30	0.08	Reference		
WT-0	ACT8	19.70	19.72	0.04	Reference		
WT-0.5	ACT8	19.45	19.47	0.03	Reference		
lcmt1-0	ACT8	22.10	22.08	0.13	Reference		
lcmt1-0.5	ACT8	20.36	20.45	0.14	Reference		