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Fakulteten för landskapsarkitektur, trädgårds- och växtproduktionsvetenskap

Effects of cold treatment on *Arabidopsis thaliana*

- A study in the change of gene expression levels in some selected COR-genes and composition of important cell membrane lipids of *A. thaliana*

Effekter av köldbehandling på *Arabidopsis thaliana*

- En studie i förändringen av nivån av genuttryck för några COR-gener och sammansättningen av viktiga cellmembranlipider hos *A. thaliana*

Artur Andersson

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Förord

Jag vill börja med att tacka min handledare Ida Lager, som har hjälpt mig med allt från upplägg till utförande av arbetet, outtröttligt svarat på mina frågor och varit väldigt positiv genom hela processen, vilket hjälpt mig enormt.

Jag vill även tacka Salla Marttila, vars föreläsning om stress hos växter, grodde fröet till mitt intresse kring membranlipiders roll för acklimatisering till nya förhållanden.

Sammanfattning

Effekten av köldstress på *Arabidopsis thaliana*, backtrav, undersöktes. Syftet var att utreda hur genuttrycket och konfigurationen av membranlipider påverkades hos fröplantor då de behandlades med låg temperatur. Enligt flera studier ändras kompositionen av fettsyror till att vara en större andel omättade hos flera viktiga cellmembranklasser vid långvarig kyla. Flera köldreglerade gener, kallade COR-gener, valdes ut, för att undersöka deras förmodade förändring i genuttrycks-nivåer efter köldbehandling. Ungefär 1000 plantor av *A. thaliana* etablerades från frö i flytande näringsmedium i tio kolvar och växte under artificiellt ljus och 20 °C under 10 dagar. Sedan behandlades hälften av fröplantorna genom att placera kolvarna på is i 6 timmar. Prover från kontroll och behandling genomgick lipidextraktion, så väl som RNA-extraktion, syntes av komplementärt DNA och kvantitativ PCR för att mäta nivåerna av genuttryck. Resultaten är inte entydiga. Beträffande cellmembranlipiderna, kunde endast uppmätas en minskning av mättnadsgraden hos fettsyrorna på 6-10% % hos vissa membranlipidklasser. Rörande nivåerna av genuttryck hos de olika köldreglerade generna, varierar dessa från att vara 1,1 gånger till 2,5 gånger större hos behandlade prover i jämförelse med kontrollprover.

Abstract

The effect of cold stress in *Arabidopsis thaliana*, mouse cress, was examined. The object was to investigate how gene expression and membrane lipid configuration was affected in seedlings when being treated with low temperature. According to several studies, the fatty acid composition changes to being less saturated in several important cell membrane lipid classes during prolonged cold. Several cold regulated; so called COR-genes were chosen, to investigate their presumed change in gene expression levels after cold treatment. Roughly 1000 plants of *A. thaliana* were established from seeds in liquid nutrient medium in ten flasks and were grown under artificial light and 20 °C for 10 days. Then half of the seedlings were treated by putting the flasks on ice for 6 hours. Control and treatment samples went through lipid extraction and gas chromatography, as well as RNA extraction, complementary DNA synthesis and quantitative PCR. The results are ambiguous. Regarding cell membrane lipids, only a minor decrease from saturated to unsaturated fatty acids of around 6-10% could be measured for some membrane lipid classes. Concerning gene expression levels, the levels vary from being 1.1 to 2.5 times greater in treated samples, compared to control samples.

Table of contents

Förord ... 3
Sammanfattning ... 4
Abstract ... 5
List of abbreviations ... 7
1. Introduction ... 8
1.1 Background ... 10
1.2 Task and object ... 11
2. Method ... 12
2.1 Establishment of seeds in liquid medium ... 12
2.2 Treatment and harvest of seedlings ... 12
2.3 Lipid extraction ... 13
2.4 Homogenization and RNA-extraction from samples ... 14
2.5 Complementary DNA synthesis and quantitative PCR ... 14
3. Results ... 16
3.1 Membrane lipids ... 16
3.2 Gene expression levels ... 21
4. Discussion ... 22
5. Conclusions ... 24
6. References ... 25
6.1 Protocols ... 27

List of abbreviations

PCR = polymerase chain reaction

PC = phosphatidylcholine

PA = phosphatidylethanolamine

PG = phosphatidylglycerol

MGDG = monogalactosyldiacylglycerol

DGDG = digalactosyldiacylglycerol

COR-genes = cold regulated genes

GPCAT = Glycerophosphocholine Acyltransferase

A-CoA-d.l.2 = Acyl-CoA desaturase-like2

NAC1 = NAC domain containing protein 1

AGP-15 = Arabinogalactan protein 15

F3H = Flavanone 3-hydroxylase

GOI = gene of interest

HKG = housekeeping gene

Cq = cycle quantification

1. Introduction

Plants have different mechanisms to cope with the surrounding conditions. Through the millions of years that plants have existed, millions and millions more have gone extinct due to their inadequate ability in adapting to their surroundings. The plants that live today are in many cases experts of dealing with the habitat in which they grow. This can partially be explained by the never-ending evolution and “survival of the fittest” theory, plants that are successful will also have a big impact on the gene pool in a population.

When being subject to external damage or conditions differing from their optima, plants experience stress, albeit to humans sometimes hardly noticeable in their morphology, which also affect their health. This stress can be biotic or abiotic. Biotic stress is defined as “a stress that is caused in plants due to damage instigated by other living organisms, including fungi, bacteria, viruses, parasites, weeds, insects, and other native or cultivated plants” (Newton et al., 2011). Abiotic stress means it is caused by some factor other than a living, such as deficiency of nutrients, toxic compounds, dehydration or flooding, lack of light or sunburn, wind, temperatures outside the “adequate zone”, and so on (Zaidi et al., 2014). Abiotic stress often leads to reduced growth, diminishing yields and in the more extreme cases impaired fertility or death.

When experiencing new conditions, a major factor in the plants success in surviving and thriving, stands their phenotypic plasticity. This, according to Taiz and Zeiger (2010), amounts to the plants ability to change its morphology or physiology in non-permanent ways when being subjected to changes in the environment. Plants need to be more adaptable to environmental fluctuation than most animals, because it is impossible for them to strictly “run away from danger”. Many studies have through the use of genome sequencing, mutational analysis and/or genetic modification showed that there are complex genetic changes occurring when plants are exposed to various treatments (Hajela et al., 1990; Purdy et al., 2011) These changes can happen in many ways, of which some are very complex and not yet fully understood.

The perhaps most important example of changes in plants in response to fluctuating environment is gene expression, which can be controlled by epigenetic, transcriptional, post-transcriptional, and post-translational mechanisms (Joshi et al., 2018). In plants, this is one of the fastest ways to adapt to abiotic stress, since changes can take place in the matter of hours and frequently even minutes. According to Blackstock (1989) “gene expression is a multistep process that involves transcription, mRNA maturation and splicing, translation and degradation of mRNAs and proteins”.

In plants, the change in gene expression can be measured using several different methods, there included the real time quantitative PCR method (Heid et al., 1996) and basically gives an inclination of how the plant responds. Genes that are normally related to stress should have low expression in the transcription of a thriving plant. On the other hand, genes that are important for normal growth and cell function should be expressed at a stable level. Measuring expression of certain genes that are interesting serves a unique possibility to gain new knowledge of biological processes during altering conditions (Ganeshan et al., 2008; Farooqi et al., 2018).

Cell membrane lipids have also been found to play an important role in plants´ abilities of adapting to abiotic stress, for example regarding temperature (cold) stress (Rudolphi-Skórska and Sieprawska, 2015; Iwaya-Inoue et al., 2018). The meaning of this process is not completely understood, but it seems that membrane lipids show a higher unsaturation when being treated with freezing or low temperature. Studies have suggested that thanks to the higher unsaturation of fatty acids, membrane lipids become less tightly packed and more “fluid” which in theory makes them more cold resistant (Upchurch, 2008; Barrero-Sicilia et al., 2017; Longo et al., 2018). This could in turn have implications of ice crystals not being able to penetrate or damage the membrane as easily (Steponkus, 1984) and/or because antifreeze proteins are prone to have a higher abundance in the membrane (Iba, 2002).

1.1 Background

This study will take a closer look on some selected COR-genes (cold regulated genes). According to Shi et al., (2017) there were over 3000 COR-genes with changed expression levels in *A. thaliana* during treatment for 12 hours at 4 °C. The same study also found the gene expression levels to be the highest after 8-12 hours, although significant change could be measured already after 4 hours.

Some genes that are related to cold tolerance were chosen to be investigated in the experiment. These include the gene called **AT5G35460**[1]; which codes for an enzyme called Glycerophosphocholine Acyltransferase (GPCAT) which has the function of adding acyl groups to Glycero-3-phosphocholine (GPC) (Lager et al., 2015; Głąb et al., 2016).

AT2G31360[2]; which encodes a protein called acyl-CoA desaturase-like2 which takes part in the synthesis of sphingolipids, the 24:1n-9 and 26:1n-9 components of seed lipids, and the membrane phospholipids phosphatidylserine (PS), and phosphatidylethanolamine (PE) (Smith et al., 2013).

AT1G01010[3]; called NAC domain containing protein 1, which has the function as a transcriptional activator that is activated upon stress by proteolytic cleavage through regulated intramembrane proteolysis (“Arabidopsis eFP Browser,” n.d.; “NAC001 - NAC domain-containing protein 1 - Arabidopsis thaliana (Mouse-ear cress) - NAC001 gene & protein,” n.d.).

AT5G11740[4], known as Arabinogalactan protein 15, which has been suggested to have a function in cell to cell signaling, possibly by carrying mRNA across the cell membrane (Guan and Nothnagel, 2004).

AT3G51240[5]; which encodes a protein called flavanone 3-hydroxylase (F3H) that regulates biosynthesis of certain flavonoids (“AT3G51240(F3H),” n.d., p. 51240).

AT3G18780[6], called actin-2 which is constitutively expressed in vegetative tissue and is involved in tip growth of root hairs (Volkov et al., 2003; “AT3G18780(ACT2),” n.d., p. 18780). The last gene is a so-called housekeeping gene (HKG)

The membrane lipids that are being investigated are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) which all make up a high proportion in the cell membrane, together with monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) which are mostly found in the thylakoid membranes in chloroplasts (Steponkus, 1984; Barrero-Sicilia et al., 2017). This far, only cell membrane lipids have been found to play a role in cold acclimation in plants, and therefore it is expected that there will be no difference in fatty acid composition regarding the lipid classes MGDG and DGDG.

1.2 Task and object

The main need for this project is the knowledge that it can provide in terms of better understanding for which mechanisms plants use to survive an ever-changing environment and further offer guidance towards subjects of interest for future studies to investigate.

The task of this project was to examine the gene expression levels and cell membrane lipid configuration in *Arabidopsis thaliana* of the ecotype Columbia-0 after cold stress treatment.

In order to try to confirm the hypothesis that changes occur in gene expression levels and lipid membrane configuration after cold stress, I used methods to measure the gene expression levels of some COR-genes, together with the composition of earlier mentioned membrane lipid classes.

The object of this paper is to present the results gained from this experiment and discussing their significance and future application.

2. Method

2.1 Establishment of seeds in liquid medium

The top of 10 flasks is covered with two layers of aluminum foil and autoclaved. One bottle is filled with about 800 ml of distilled water and 2.165 grams of MS nutrient solution is added. pH is set to 5.7. The bottle is filled up to 900 ml, which is distributed evenly between two bottles which are then autoclaved. A solution of 40 g sucrose and 200 ml distilled water is mixed in a bottle, this solution then goes through a pressurized sterilization filtration. 100 ml of the sucrose solution is added to the now autoclaved nutrient solution.
On sterile lab bench:
50 mg seeds of *Arabidopsis thaliana* of ecotype Columbia-0 is treated with 70% EtOH and shaken for 1 min by hand. EtOH is then removed by pipetting. The seeds are then transferred to a solution of 500 µl sterile water, 500 µl sodium hypochlorite and 5 µl Tween-20 and are shaken for 5 min by hand. This solution is then pipetted away, and the seeds are rinsed in sterile water. The seeds are transferred to a Falcon tube, thereafter two drops of 40% glycerol are added, and water up to 2 ml. The liquid medium is distributed between the ten flasks. 200 µl of the seed solution is transferred to every flask. The flasks are then put in a climate room at 20°C and artificial light for 10 days, standing on a shaker.

2.2 Treatment and harvest of seedlings

Five of the ten flasks are subjected to cold treatment for 6 hours. The flasks are placed on ice, which is changed roughly once every two hours, to keep the temperature down to 0 °C. The other five flasks are acting control and are not subjected to any treatment.
After 6 hours the seedlings (roots, stems and leaves) are taken out of the flasks (the flasks stand on ice until the seedlings are harvested) and washed thoroughly and quickly in milli-Q-water. The seedlings have formed a kind of “lump” that is cut apart in two pieces - of which one part is used for lipid extraction and one part is used for

RNA-extraction later on - dried with paper and placed in two different Falcon tubes which are then directly submerged in liquid nitrogen. This is then repeated for all the treated samples and the control samples. All the RNA samples are then frozen at -80°C. The lipid samples are freeze-dried for two days.

2.3 Lipid extraction

Lipids are extracted from the freeze-dried samples according to the protocol “Lipid och fettsyrasammansättning i olika växtvävnader”; doing the following steps. The fresh weight is measured for all samples and then transferred to individual glass-homogenizers. 1 ml of 0.15 M HAc and 3.75 ml of MeOH:$CHCl_3$ (2:1) is added. The samples are homogenized. The liquid is poured over into a glass tube with a lid and the glass-homogenizer is washed with 1.25 ml $CHCl_3$ which is then poured into the glass tube. 1.25 ml of H_20 is added and then the glass tube is vortexed shortly and centrifugated at 2000 rpm for 2 minutes. Unfortunately, the samples weren’t marked during the centrifugation, and therefore it was impossible to calculate the value of lipids per dry weight in individual samples (although it was possible to differentiate treated samples from control samples because of the orientation of which they were put in). The lowest liquid phase ($CHCl_3$-phase) of the tube is then transferred to a new glass tube and put in the freezer at -20°C overnight.
Lipid extraction is completed following the same protocol. 1 ml of each sample is transferred to an evaporation glass tube with a hollow lid and put on hot sand where the liquid phase evaporates. The lipids at the bottom are then redissolved in 25 µl $CHCl_3$. A TLC (Thin Layer Chromatography) silica gel plate is loaded with the diluted lipids, together with two lipid standards. The plate is placed in a glass tank with polar medium $CHCl_3$:MeOH:HAc:H_2O (90:15:10:3 ml ratio) during 90 min. The plate dried for 5 minutes and was then sprayed with primulin. The lipid classes on the plate are visualized using UV-light, marked out and some are then scraped off and placed into a glass tube with screw cap and covered with methanol. The silica is dried on hot sand, then 2 ml 2% H_2SO_4 in MeOH is added and incubated at 90°C for 30 min. The samples are then cooled off to room temperature and an internal standard of 17:0 methyl ester is added to every tube, together with heptane and milli-Q-water. The tubes are then briefly vortexed and centrifuged for 2 min at 2000 rpm. The upper

phase is then transferred to an evaporation tube and put on hot sand. When the liquid has evaporated, 150 µl of heptane is added. The lipid samples are stored in the freezer at -20°C overnight. The samples are transferred to GC-vials and then run on Gas chromatography overnight.

2.4 Homogenization and RNA-extraction from samples

The RNA samples are taken out of the freezer and homogenized in a milling machine for 30 sec; the steel container has been chilled in liquid nitrogen beforehand. The powder is transferred to a cooled Eppendorf tube and instantly submerged in liquid nitrogen. Only three of the controls respectively treatments are chosen; control 1, 2 and 3 (called K1, K2 and K3), together with treatment 2, 3 and 5 (called B2, B3, and B5). RNA is extracted from the samples following the protocol “PureLink™ Plant RNA Reagent” (“PureLink Plant RNA Reagent,” n.d.). Five small spoons of frozen, ground tissue is used from each sample. After extraction, the samples are analyzed on an Xpose spectrophotometer. RNA extracts are loaded onto an e-gel to confirm their quality. Genomic DNA is degraded from the samples by following the protocol “DNase, RNase-free, Thermo Scientific” (“User Guide: DNase I, RNase-free, 1U/uL,” n.d.).
Primers are resuspended to a concentration of 100 µM using milli-Q-water and then diluted to a concentration of 10 µM in Eppendorf tubes (using 20 µl 100 µM primer solution and 180 µl milli-Q-water). The RNA extracts are stored in the freezer.

2.5 Complementary DNA synthesis and quantitative PCR

Following the protocol of “Thermo Scientific Maxima First Strand cDNA Synthesis kit for RT-qPCR” (“User Guide: Maxima First Strand cDNA Synthesis Kit RT-qPCR w dsDNase, K1672,” n.d.) RNA is converted to complementary DNA (cDNA). Quantitative PCR was done following the protocol of “Maxima SYBR Green/ROX qPCR Master Mix (2X)” (“User Guide: Maxima SYBR Green/ROX qPCR Master Mix, k0221,” n.d.). For each primer pair; 3 wells of cDNA, 3 wells of background (-RT) from sample control 3 (K3) and two wells of water was included. The plates run on

PCR for 97 minutes (40 cycles). The best primers were chosen, based on the lowest Cycle quantification values and showing single melt curves.

To check that the chosen primers are amplifying in a linear fashion over different complementary DNA concentrations, different concentrations of cDNA (1:1, 1:10, 1:100) together with milli-Q-water were loaded onto a plate and went through PCR.

Gene	Primer	Primer sequence (5'–3')	Primer efficiency (%)
Glycerophosphocholine acyltransferase (AT5G35460)	Gene 1- forward	TTGTTTCCTACTTGGTGCAAGACC	95.0
	Gene 1- reverse	TGGCATAGTAGCAAAAGTCCAGA	
Acyl-CoA -like desaturase 2 (AT2G31360)	Gene 2- forward	CATCAACGGTGGAGGAGAAC	99.5
	Gene 2- reverse	GGAGCCAAGAGAGCAAGAGA	
NAC-domain containing protein (AT1G01010)	Gene 3- forward	TCAGCGAGGTCAACATCTGT	99.9
	Gene 3- reverse	TTACCACGAAAGCCCTCACT	
Arabinogalactan-15 (AT5G11740)	Gene 4- forward	ACCCCTCACCACATTTCATGA	99.9
	Gene 4- reverse	GACGCTGAGATCGCACTAGA	
Flavanone 3-hydroxylase (AT3G51240)	Gene 5- forward	GGAGCAATATCAGCCGTCGA	96.5
	Gene 5- reverse	GGAAGCCTTAGAGATCGCCA	
Actin-2 (AT3G18780)	Gene 6- forward	TCTTAACCCAAAGGCCAACA	97.0
	Gene 6- reverse	CAGAATCCAGCACAATACCG	

Table 1. Information about primer pairs; primer sequence and primer efficiency calculated from plate values.

All chosen samples were then loaded onto plates (3 replicate wells of cDNA, 3 wells of background-RT, and 2 wells of milli-Q-water for each primer pair and individual sample) and went through quantitative real time PCR.

The results were assessed using software Thermo Fisher Connect.

3. Results

3.1 Membrane lipids

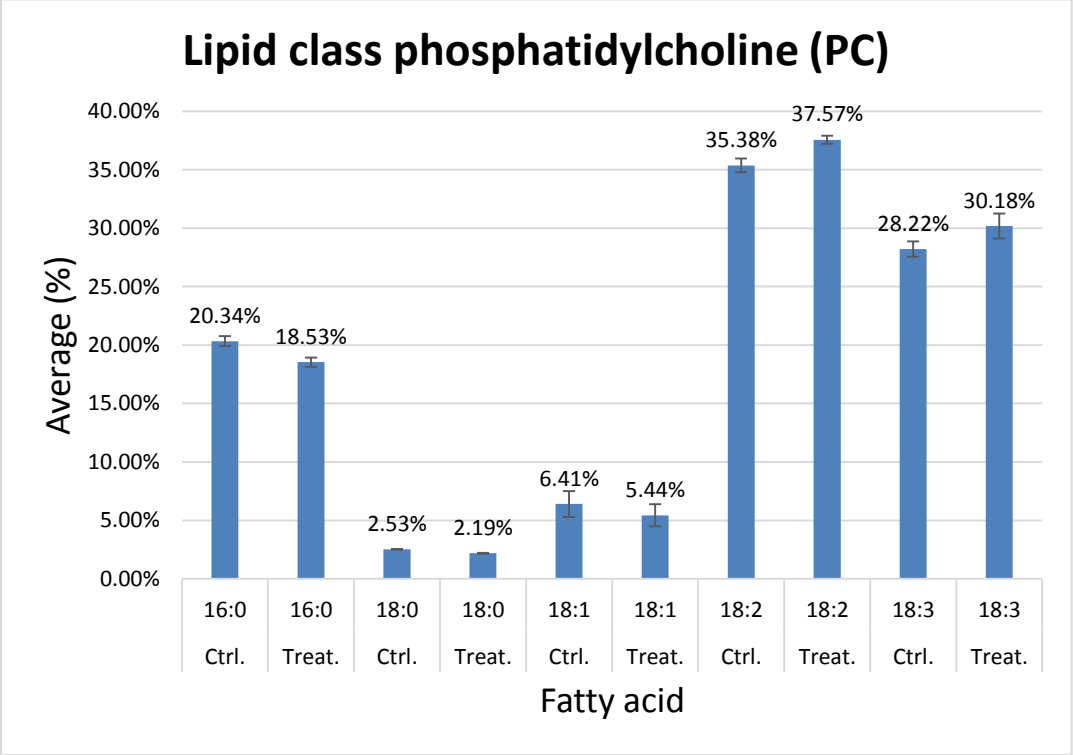

Chart 1. The average content (in % of total lipids) of different fatty acids for all controls and treatments in the membrane lipid class phosphatidylcholine (PC). Ctrl. stands for control samples. Treat. stands for treated samples. The bars show individual standard deviation values from the average. Only fatty acids that make up 2% or more of the total in any sample are accounted for.

The results show an 8.9% decrease of the 16:0 fatty acid content of lipid class PC, between control and treatment. This might seem quite large, but the variation between different samples of both control and treated samples is almost as high, therefore the difference is not significant. No fatty acid with 16 carbon atoms - other than 16:0 - made up 2% or more in any sample. A minor decrease is also visible in the 18:0 fatty acid content, 14.4%. This is probably the most certain change of fatty acid content, since the difference between individual samples are pretty much zero, as visible from the standard deviation bars. A slight increase is visible in the fatty acid 18:2 and 18:3, 6.1% and 6.9% respectively. This increase is on the same confidence level as the decrease of the 16:0 fatty acid.

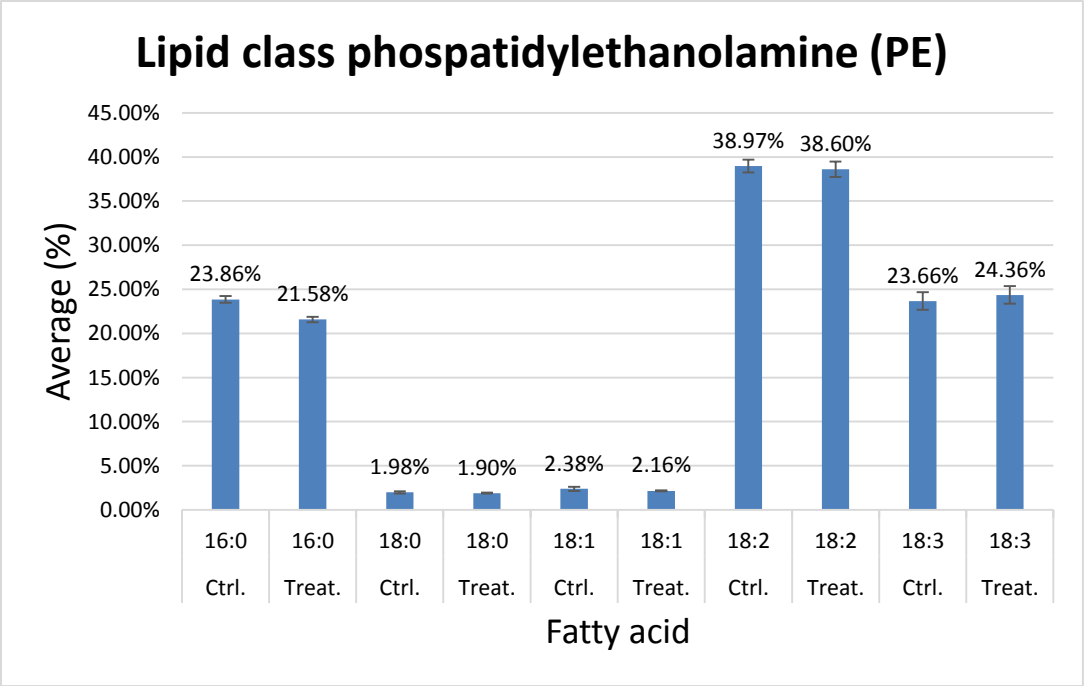

Chart 2. The average content (in % of total lipids) of different fatty acids for all controls and treatments in the lipid class phospatidylethanolamine (PE). Ctrl. stands for control samples. Treat. stands for treated samples. The bars show individual standard deviation values from the average. Only fatty acids that make up 2% or more of the total in any sample are accounted for.

The results for the lipid class PE show a decrease in 16:0 fatty acid content of 9.6%. This is in parity with the result gained from the lipid class PG. The change is almost invisible between control and treated samples regarding the 18:0 and 18:1 fatty acids, as it is concerning the 18:2 and 18:3 fatty acids, with quite high standard deviations.

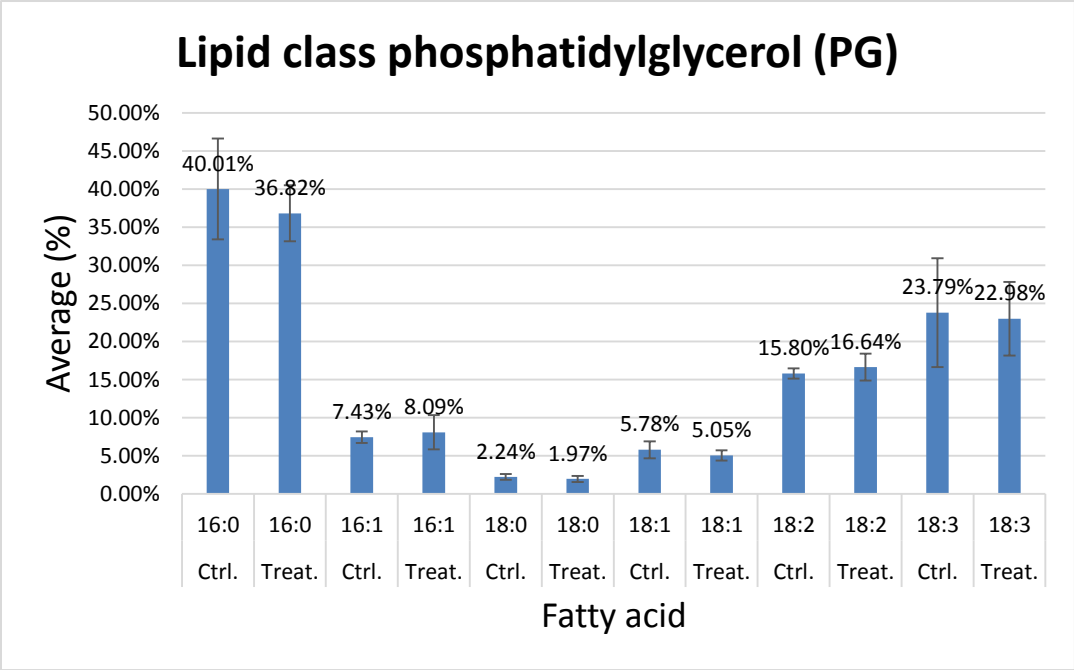

Chart 3. The average content (in % of total lipids) of different fatty acids for all controls and treatments in the lipid class phosphatidylglycerol (PG). Ctrl. stands for control samples. Treat. stands for treated samples. The bars show individual standard deviation values from the average. Only fatty acids that make up 2% or more of the total in any sample are accounted for.

The results for the lipid class PG show a decrease of fatty acid 16:0 at 8.0%, but with very high standard deviation. This is also the case when looking at all values gained from the lipid class PG, the variation between samples is high and possible changes between control and treated samples cannot be fortified in any way.

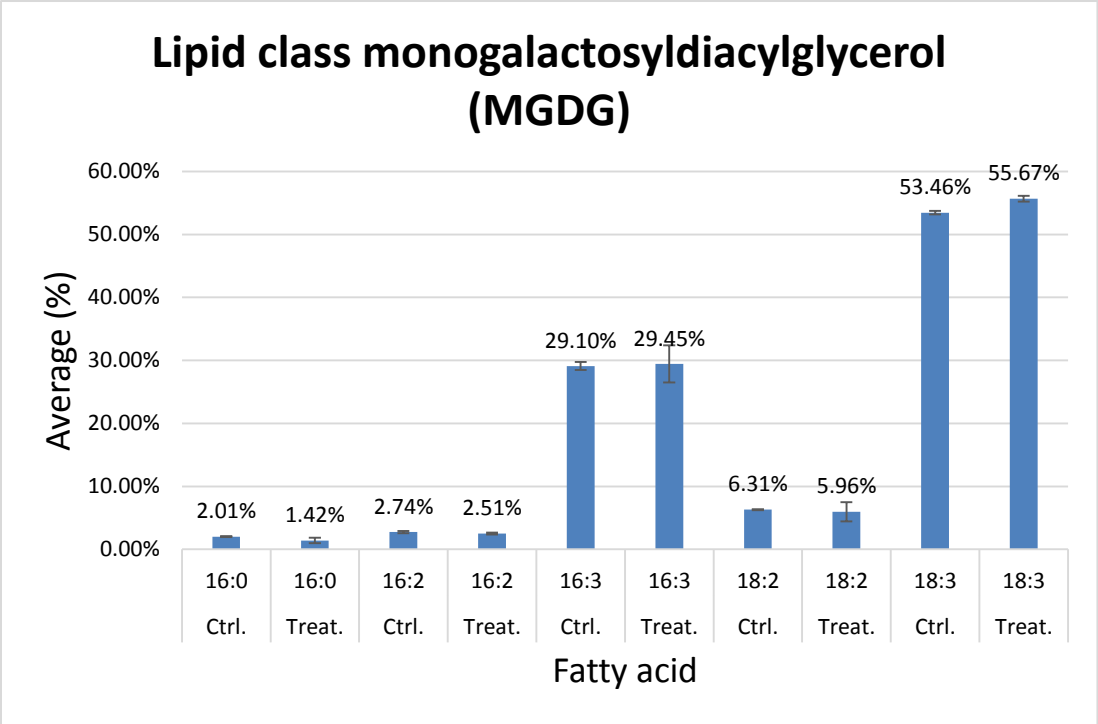

Chart 4. The average content (in % of total lipids) of different fatty acids for all controls and treatments in the lipid class monogalactosyldiacylglycerol (MGDG). Ctrl. stands for control samples. Treat. stands for treated samples. The bars show individual standard deviation values from the average. Only fatty acids that make up 2% or more of the total in any sample are accounted for.

The results gained from lipid class MGDG show an increase in the content of fatty acid 16:0, 16:2 and 18:2. The changes are small and have a low standard deviation for these fatty acids, which seem to fortify the hypothesis that the lipid class MGDG does not have any implication on cold acclimation. The only values that are not expected are the ones gained for fatty acid 18:3, where an increase of 4.1% is visible, while showing a low standard deviation.

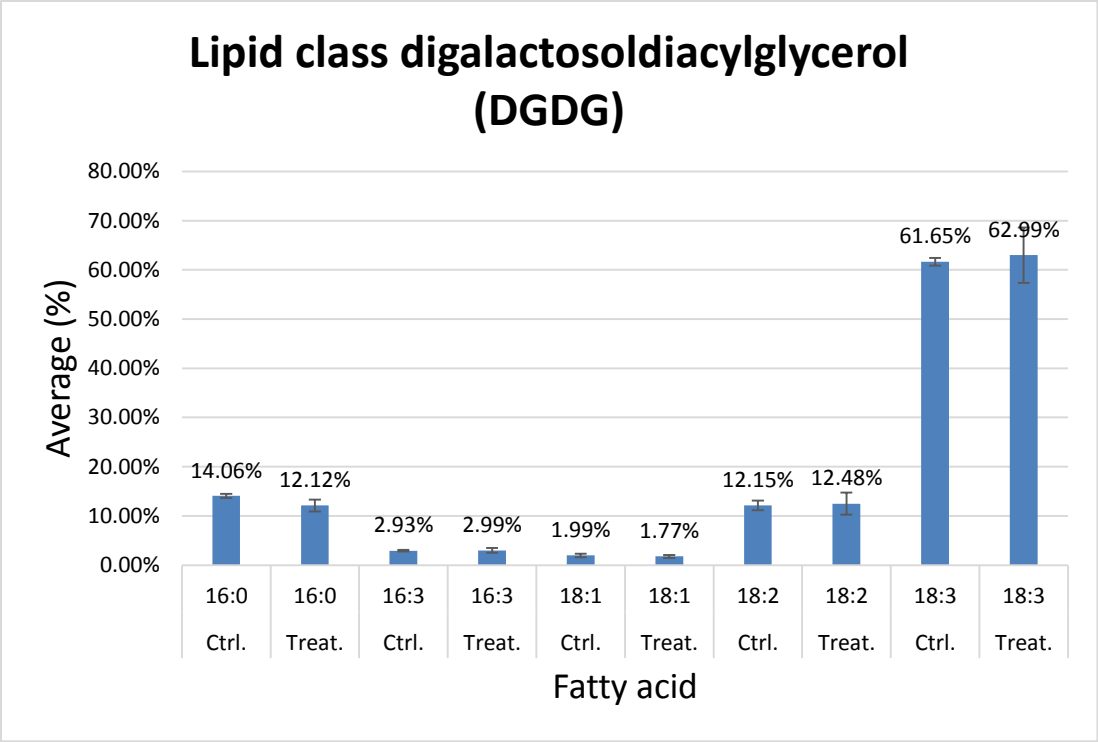

Chart 5. The average (in % of total lipids) of different fatty acids for all controls and treatments in the lipid class digalactosoldiacylglycerol (DGDG). Ctrl. stands for control samples. Treat. stands for treated samples. The bars show individual standard deviation values from the average. Only fatty acids that make up 2% or more of the total in any sample are accounted for.

The results gained for lipid class DGDG are in parity of the ones gained for the lipid class MGDG. This also seems to cohere with the theory that the lipids of the thylakoid membrane do not have much to do with cold acclimation in plants.

3.2 Gene expression levels

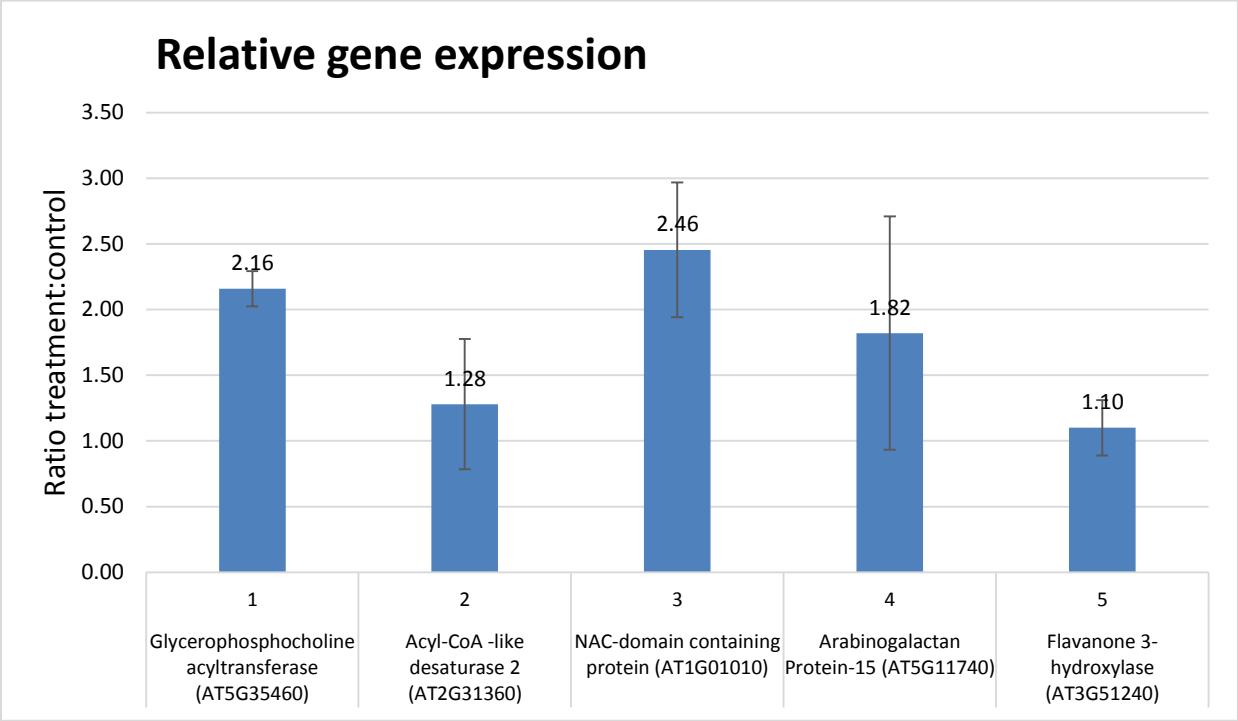

Chart 6. The ratio of gene expression levels between treatment and control samples for all the genes investigated. The error bars show individual standard deviation values. Gene expression levels and ratios were calculated using the Pfaffl method ("How To Perform The Pfaffl Method For qPCR," n.d.). Cq-levels were set to 40 (maximum cycles) in wells where the threshold value was not reached during PCR.

The values gained from gene expression levels show an increase of transcription in every gene of interest between treatment and control, with varying ratios from 1.10 for gene F3H to 2.46 for gene NAC1. The ratios are quite low for all genes, and do not strictly point to the conclusion that the plants have actually experienced cold stress, since gene expression levels can be different between different plants in the same conditions. The genes exhibit quite high standard deviation values, which directly excludes the genes A-CoA-d.l.2, AGP-15 and F3H because the gene expression levels then reach the same ratio of the treated samples as for the control samples. The genes GPCAT and NAC1 still show an increase when regarding standard deviation error values.

4. Discussion

The results do not show any statistically significant change in the configuration of membrane lipids, for any lipid class. This was expected for the two lipid classes that are not present in the cell membrane, but instead the thylakoid membrane, MGDG and DGDG. These have not been shown to play a part in cold acclimation in earlier studies made either. There is a slight difference in the configuration of fatty acids in the lipid classes PG, PE and PC, but not adequate to draw any conclusions from it. The change for these lipid classes between saturated and unsaturated fatty acids was in the range of 2-3%. These minor changes in results can be explained by many factors. Perhaps the single most deciding factor is the treatment itself. The flasks with seedlings were put on ice for six hours; perhaps the seedlings did not experience quite the ice-cold treatment but instead only a slight change in temperature from the start, because of the spatial barrier that the liquid medium provided. Another big factor is probably the time under which the treatment proceeded. From information taken from other studies, 4 hours would be well enough to measure significant change in the configuration of certain membrane lipid classes in both roots and leaves. This would be true for a single plant that is put in a room where the air is +4°C or lower, thus this treatment was different. Here, the seedlings were in a kind of lump, possibly protecting each other, and the air was probably not chilled to 0°C, since the cold source came from underneath. Another factor of interest could be the age of the seedlings, in several earlier studies plant were at least 2 weeks old and grown individually. In this experiment, the seedlings were only 10 days old and perhaps differed in some developmental aspect. Another possible source of error might be the choosing of the whole seedling to examine. This differs from earlier studies referenced here, where only “above ground” parts have been examined. The roots might have a different way of dealing with cold stress, which seems reasonable regarding the fact that they are never really exposed to very low or high temperature the way stems and leaves and all other above ground parts are. The roots therefore might influence the gained results, especially regarding fatty acid composition.

Regarding the results of the gene expression levels of the COR-genes investigated, not many conclusions can be drawn. The ratio between treatment and control ranges

from 1.10 to 2.46. The only gene which was not earlier clearly related to higher gene expression levels during cold treatment was A-CoA-d.l.2. This gene showed a ratio of 1.28, which is not very high. The sources of error regarding the very different results that was showed in regard to expected levels - are many. One of the factors that most likely did not have any negative influence on the results is the primer pair efficiency, which were within recommended range, varying from 95-99%. Another factor that most likely did not affect the results is the RNA extraction process, which in the Xpose spectrophotometer showed the RNA quantity to vary from 1432.5 to 1796.2 ng/µl; and with very little contamination from DNA. This is well enough to use in complementary DNA synthesis and should not have any influence on the unexpected results. The biggest factor that most likely does have a big importance in giving these results is the cDNA synthesis. The wells for samples K2, B2 and B5 in general had nonexistent or very high Cq-values. The Cq-values for K1, K3 and B3 are also high in general. This is an inclination that the cDNA synthesis was not successful enough to give the amounts needed for primers to amplify the genes of interest to reach the threshold value during the time of PCR. The cDNA synthesis also affects the results more so than other factors, because it gives very differing results of the house keeping gene expression levels, which in turn are used to calculate the expression levels of all genes of interest. Another factor of uncertainty is the freeze/thaw cycles of cDNA. The samples were stored for several days at -20°C, which in itself should not be a problem, with good stability for up to one week. The influencing factor could be the freeze, thaw cycle that happened every time a new plate needed to be loaded with cDNA. In the extension of the experiment, the cDNA had gone through several of these cycles, which could in turn influence the integrity of the cDNA.

The genes (except for GPCAT and A-CoA-d.l.2) are not known to be directly related to the change in membrane lipid configuration, and therefore the low ratios of gene expression levels in the rest of the genes should only be partly explained by the same error sources as for the membrane lipid results. In general, gene expression changes much quicker than the configuration of membrane lipids and is therefore more likely to change, even *if* the treatment was not as effective as it ought to be. Another factor pointing to the treatment not being the major cause for error regarding gene expression levels, are the results of Cq-values gained from samples K1, K3 and

B3. These samples present significant change in expression levels for several genes, but when calculating the average of all samples, the results are not conclusive.

5. Conclusions

It is very difficult to draw any conclusions from the results, being that they are not very clear. Regarding the membrane lipid configuration, slight change is visible and could be theorized to be different from treatment to control, but not much so when coming to draw the conclusion that it has actually changed. The gene expression level ratios between treatment and control are very small, not big enough to be certain of any actual change of gene expression.

In future studies certain safety nets should be employed, with a larger number of control and treatment samples. The sources of errors in this specific study are hard to pinpoint, and therefore the method and results showed should be regarded with certain amounts of skepticism.

6. References

Arabidopsis eFP Browser [WWW Document], n.d. URL http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (accessed 5.19.20).

AT3G18780(ACT2) [WWW Document], n.d. URL https://www.arabidopsis.org/servlets/TairObject?id=39062&type=locus (accessed 5.6.20).

AT3G51240(F3H) [WWW Document], n.d. URL https://www.arabidopsis.org/servlets/TairObject?id=36753&type=locus (accessed 5.6.20).

Barrero-Sicilia, C., Silvestre, S., Haslam, R.P., Michaelson, L.V., 2017. Lipid remodelling: Unravelling the response to cold stress in Arabidopsis and its extremophile relative Eutrema salsugineum. Plant Sci. 263, 194–200. https://doi.org/10.1016/j.plantsci.2017.07.017

Blackstock, J.C., 1989. CHAPTER 17 - Gene expression, in: Blackstock, J.C. (Ed.), Guide to Biochemistry. Butterworth-Heinemann, pp. 208–221. https://doi.org/10.1016/B978-0-7236-1151-6.50023-6

Farooqi, M.Q.U., Zahra, Z., Lee, J.K., 2018. Molecular Genetic Approaches for the Identification of Candidate Cold Stress Tolerance Genes, in: Wani, S.H., Herath, V. (Eds.), Cold Tolerance in Plants: Physiological, Molecular and Genetic Perspectives. Springer International Publishing, Cham, pp. 37–51. https://doi.org/10.1007/978-3-030-01415-5_2

Ganeshan, S., Vitamvas, P., Fowler, D.B., Chibbar, R.N., 2008. Quantitative expression analysis of selected COR genes reveals their differential expression in leaf and crown tissues of wheat (Triticum aestivum L.) during an extended low temperature acclimation regimen. J. Exp. Bot. 59, 2393–2402. https://doi.org/10.1093/jxb/ern112

Głąb, B., Beganovic, M., Anaokar, S., Hao, M.-S., Rasmusson, A.G., Patton-Vogt, J., Banaś, A., Stymne, S., Lager, I., 2016. Cloning of Glycerophosphocholine Acyltransferase (GPCAT) from Fungi and Plants. J. Biol. Chem. 291, 25066–25076. https://doi.org/10.1074/jbc.M116.743062

Guan, Y., Nothnagel, E.A., 2004. Binding of Arabinogalactan Proteins by Yariv Phenylglycoside Triggers Wound-Like Responses in Arabidopsis Cell Cultures. Plant Physiol. 135, 1346–1366. https://doi.org/10.1104/pp.104.039370

Hajela, R.K., Horvath, D.P., Gilmour, S.J., Thomashow, M.F., 1990. Molecular Cloning and Expression of cor (Cold-Regulated) Genes in Arabidopsis thaliana. Plant Physiol. 93, 1246–1252. https://doi.org/10.1104/pp.93.3.1246

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Res. 6, 986–994. https://doi.org/10.1101/gr.6.10.986

Iba, K., 2002. ACCLIMATIVE RESPONSE TO TEMPERATURE STRESS IN HIGHER PLANTS: Approaches of Gene Engineering for Temperature Tolerance. Annu. Rev. Plant Biol. 53, 225–245. https://doi.org/10.1146/annurev.arplant.53.100201.160729

Iwaya-Inoue, M., Sakurai, M., Uemura, M. (Eds.), 2018. Survival Strategies in Extreme Cold and Desiccation: Adaptation Mechanisms and Their Applications, Advances in Experimental Medicine and Biology. Springer Singapore, Singapore. https://doi.org/10.1007/978-981-13-1244-1

Joshi, R., Singh, B., Chinnusamy, V., 2018. Genetically Engineering Cold Stress-Tolerant Crops: Approaches and Challenges, in: Wani, S.H., Herath, V. (Eds.), Cold Tolerance in Plants: Physiological, Molecular and Genetic Perspectives. Springer International Publishing, Cham, pp. 179–195. https://doi.org/10.1007/978-3-030-01415-5_10

Lager, I., Glab, B., Eriksson, L., Chen, G., Banas, A., Stymne, S., 2015. Novel reactions in acyl editing of phosphatidylcholine by lysophosphatidylcholine transacylase (LPCT) and acyl-CoA:glycerophosphocholine acyltransferase (GPCAT) activities in microsomal preparations of plant tissues. Planta 241, 347–358. https://doi.org/10.1007/s00425-014-2184-1

Longo, V., Janmohammadi, M., Zolla, L., Rinalducci, S., 2018. What Can Small Molecules Tell Us About Cold Stress Tolerance in Plants?, in: Wani, S.H., Herath, V. (Eds.), Cold Tolerance in Plants: Physiological, Molecular and Genetic Perspectives. Springer International Publishing, Cham, pp. 127–157. https://doi.org/10.1007/978-3-030-01415-5_8

NAC001 - NAC domain-containing protein 1 - Arabidopsis thaliana (Mouse-ear cress) - NAC001 gene & protein [WWW Document], n.d. URL https://www.uniprot.org/uniprot/Q0WV96-1 (accessed 5.6.20b).

Newton, A.C., Johnson, S.N., Gregory, P.J., 2011. Implications of climate change for diseases, crop yields and food security. Euphytica.

Purdy, S.J., Bussell, J.D., Nelson, D.C., Villadsen, D., Smith, S.M., 2011. A nuclear-localized protein, KOLD SENSITIV-1, affects the expression of cold-responsive genes during prolonged chilling in Arabidopsis. J. Plant Physiol. 168, 263–269. https://doi.org/10.1016/j.jplph.2010.07.001

Rudolphi-Skórska, E., Sieprawska, A., 2015. Physicochemical techniques in description of interactions in model and native plant membranes under stressful conditions and in physiological processes. Acta Physiol. Plant. 38, 22. https://doi.org/10.1007/s11738-015-2034-1

Shi, Y., Huang, J., Sun, T., Wang, X., Zhu, C., Ai, Y., Gu, H., 2017. The precise regulation of different COR genes by individual CBF transcription factors in Arabidopsis thaliana. J. Integr. Plant Biol. 59, 118–133. https://doi.org/10.1111/jipb.12515

Smith, M.A., Dauk, M., Ramadan, H., Yang, H., Seamons, L.E., Haslam, R.P., Beaudoin, F., Ramirez-Erosa, I., Forseille, L., 2013. Involvement of Arabidopsis ACYL-COENZYME A DESATURASE-LIKE2 (At2g31360) in the Biosynthesis of the Very-Long-Chain Monounsaturated Fatty Acid Components of Membrane Lipids. Plant Physiol. 161, 81–96. https://doi.org/10.1104/pp.112.202325

Steponkus, P.L., 1984. Role of the Plasma Membrane in Freezing Injury and Cold Acclimation. Annu. Rev. Plant Physiol. 35, 543–584. https://doi.org/10.1146/annurev.pp.35.060184.002551

Taiz, L., 2010. Plant physiology, 5. ed. ed. Sinauer Associates, Sunderland, MA.

Upchurch, R.G., 2008. Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. Biotechnol. Lett. 30, 967–977. https://doi.org/10.1007/s10529-008-9639-z

Volkov, R.A., Panchuk, I.I., Schöffl, F., 2003. Heat-stress-dependency and developmental modulation of gene expression: the potential of house-keeping genes as internal standards in mRNA expression profiling using real-time RT-PCR. J. Exp. Bot. 54, 2343–2349. https://doi.org/10.1093/jxb/erg244

Zaidi, N.W., Dar, M.H., Singh, S., Singh, U.S., 2014. Chapter 38 - Trichoderma Species as Abiotic Stress Relievers in Plants, in: Gupta, V.K., Schmoll, M., Herrera-Estrella, A., Upadhyay, R.S., Druzhinina, I., Tuohy, M.G. (Eds.), Biotechnology and Biology of Trichoderma. Elsevier, Amsterdam, pp. 515–525. https://doi.org/10.1016/B978-0-444-59576-8.00038-2

6.1 Protocols

How To Perform The Pfaffl Method For qPCR [WWW Document], n.d. URL https://toptipbio.com/pfaffl-method-qpcr/ (accessed 5.15.20).

PureLink Plant RNA Reagent [WWW Document], n.d. URL https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Fplantrna_man.pdf&title=UHVyZUxpbmsgUGxhbnQgUk5BIFJlYWdlbnQ= (accessed 5.14.20).

User Guide: DNase I, RNase-free, 1U/uL [WWW Document], n.d. URL https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0012000_DNase_I_RNasefree_1UuL_UG.pdf&title=VXNlciBHdWlkZTogRE5hc2UgSSwgUk5hc2UtZnJlZSwgMVUvdUw= (accessed 5.14.20).

User Guide: Maxima First Strand cDNA Synthesis Kit RT-qPCR w dsDNase, K1672 [WWW Document], n.d. URL https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0012952_Maxima_FirstStrand_cDNA_Syn_RT_qPCR_dsDNase_K1672_UG.pdf&title=VXNlciBHdWlkZTogTWF4aW1hIEZpcnN0IFN0cmFuZCBjRE5BIFN5bnRoZXNpcyBLaXQgUlQtcVBDUiB3IGRzRE5hc2UsIEsxNjcy (accessed 5.18.20).

User Guide: Maxima SYBR Green/ROX qPCR Master Mix, k0221 [WWW Document], n.d. URL https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0012924_Maxima_SYBR_GreenROX_qPCR_MasterMix_k0221_UG.pdf&title=VXNlciBHdWlkZTogTWF4aW1hIFNZQlIgR3JlZW4vUk9YIHFQQ1IgTWFzdGVyIE1peCwgazAyMjE= (accessed 5.14.20).