The improvement of chilling tolerance in chilling sensitive basil (*Ocimum basilicum* var Marian)

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Author's Declaration

I declare that the work carried out in this PhD thesis was in accordance with the rules and regulations of the Royal Holloway University of London. I declare that the work is original except where the text is cited and fully referenced and no part of this dissertation has been submitted for any other degree.

Any views expressed in this dissertation are those of the author and in no way represent those of the Royal Holloway University of London.

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Abstract

Basil is the most popular culinary herb in the UK but sensitive to chilling temperatures. Chilled basil leaves wilt and become discoloured, thus, become unappealing to consumers. Therefore, herbs are rarely maintained as part of the 'cool-chain', and this can increase the risk of microbial contamination. The aim of the project was to modify the growing conditions of basil to improve its tolerance towards chilling temperatures. Basil's sensitivity towards chilling temperatures was assessed by quantifying cell damage in several ways. Damage to membranes (membrane leakage), photosystems (chlorophyll fluorescence) and a decline in antioxidant content was 33%, 50%, and 33%, respectively after exposure of 24 h at 4°C. Basil also showed an increased lipid peroxidation (66%) and accumulation of reactive oxygen species (ROS) (85%). 3000 transcripts from the RNA sequencing experiment of basil treated with or without chilling temperature were analysed. 1206 (40.2%) of the genes were differentially regulated among which 138 (11.44%) were upregulated and 1062 (88%) downregulated in response to cold. Genes involved in metabolite and ion transport and those involved in primary metabolism (lipid, carbohydrate and ascorbate-glutathione system) were downregulated and were associated with either the chloroplast or the plasma membrane. Suppression of important metabolic pathways and defense against the generation of toxic ROS to the cell is considered a possible mechanism in basil's susceptibility towards chilling temperatures.

Light is required for the activation of cold regulated genes (COR) and the phytochromes are known to be involved in a plant's response to chilling temperatures. In this project, basil was treated with low R:FR light on four consecutive days and then exposed to 24 h of chilling stress that resulted in basil showing reduced damage to membranes, accumulation of ROS and damage to the light absorbing photosynthetic apparatus. LR:FR light treated plants also induced expression of genes coding for phyA, abscisic ABA signalling and jasmonate JA signalling or involved in scavenging of ROS that may promote basil cells survival under chilling stress.

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ABA	Abscisic acid
ABC	ATP-binding cassette transporter
ANOVA	analysis of variance
At	*Arabidopsis thaliana*
ATP	Adenosine Tri-phosphate
BLASTn	Nucleotide basic local alignment search tool
CAMTA3	CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 3
CBF	CaM-binding transcription activator
CBL	Calcineurin B-like proteins
CCAMK	Ca^{2+}-and Ca^{2+}/CaM-dependent protein kinase
cDNA	Complementary DNA
CDPK	Ca^{2+}-dependent protein kinases
COR	Cold regulated genes
DAB	3, 3'-diaminobenzidine
DAVID	Database for Annotation, Visualization and Integrated Discovery
DGE	Differential gene expression
DNA	Deoxyribonucleic acid
FASTQc	Fast quality control check
FPKM	TFragments per Kilobase of transcript per Million
FRAP	Ferric reducing ability of plasma
HSP70	Heat shock protein 70
HSP81	Heat shock protein 81
JA	Jasminc acid
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LHC	Light harvesting complex
LHC	Light harvesting complex
LHY	Late elongated hypocotyl
MAPK	Mitogen-activated protein kinase
MDA	Malondealdehyde
MGDG	Monogalactosyldiacylglycerol
NPGAP-DH	Non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase
PEPC	phospho-enol-pyruvate carboxylase
PIP	Plasma membrane intrinsic proteins
PSII	Photosystem II
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNAseq	RNA sequencing
ROS	Reactive oxygen species
RWC	Relative water content
TAIR	The Arabidopsis Information Resource
TBA	2-Thiobarbituric acid
TBF	TREF base fertiliser
TIP	Tonoplast intrinsic proteins
t-RNA	Transfer ribonucleic acid

1 INTRODUCTION

1.1 The properties of basil.

The scientific name of the herb sweet basil is *Ocimum basilicum* where the genus name Ocimum is derived from the Greek word 'to be fragrant'[1] and was given by Linnaeus in 1753[1]. Basil is one of the most popular fresh herbs used throughout the world[2,3]. Basil is native to Asia (India, Pakistan, Iran and Thailand) and can also grow in tropical and sub-tropical regions[1]. Basil has been referred to as "the king of the herbs" due to its greater popularity compared to the other herbs[1]. It was also given importance in the past when it was used to represent various beliefs in different countries. For instance, Italy used basil as a symbol of love[1,4] while Hindus believed in being buried with a leaf of basil would grant them entry into paradise and Greeks used it to represent poverty and hatred[1].

Plants produce volatile organic compounds called essential oils which play roles in defense, communication and pollinator attraction[5]. Essential oils such as a-terpenol, α-linalool, eucalyptol and α-pinene consist of antibacterial activity and antioxidative effects[6]. When measured by gas spectrophotometry, the essential oils' yield in the flowers, leaves and stem of basil was 0.5%, 1.0% and 0.05% (v/w), respectively [7]. Hence, the essential oils are commonly extracted from flowers and leaves of sweet basil through the process of steam distillation and used in the production of cosmetic, hygienic products, perfumes, dental and other oral products [8]. Different cultivars of basil can have different chemotypes where the chemical composition of its total content of essential oils may differ from one another. It is the content and the chemical composition of the essential oil that can give each cultivar of basil a distinct aroma and taste. According to Hiltunen and Holm[8] India is the largest producer of basil oils (15 tonnes) followed by Bulgaria (7 tonnes) and Egypt (5 tonnes). Essential oil of sweet basil produced by Egypt and Europe is considered to be of the highest quality and finest odour [8]. When analysed by gas spectrophotometry, its chemical composition was rich in linalool, and methyl chavicol compounds [8].

Basil was traditionally used for medicinal purposes such as in the treatment of headaches, coughs, diarrhoea, constipation, warts, worms and externally applied as an ointment to reduce skin acne [9]. Basil can also produce compounds including xanthophylls, carotenoids, zeaxanthin and phenolic compounds with antioxidative activity that makes its use suitable for medicinal purposes[10,11]**.** Antioxidants can be any substance capable of inhibiting oxidation when present in the concentration lower than that of the oxidants themselves. Various fruits and vegetables can synthesize and supply essential compounds that function as antioxidants[12], and their intake in the diet has been associated with significant protection against chronic diseases[13]. In a study by Ben-Ali *et al.*[14] the effectiveness of basil's antioxidants in inhibiting oxidation and thereby stabilising sunflower oil was investigated. The study showed the effectiveness of antioxidants from basil extracts to be higher than the commonly used synthetic antioxidant molecule butylated hydroxytoluene (BHT) in food[14]. In addition, β - carotene is the most important vitamin A precursor with antioxidative activity, and its content in basil (104.9 µg/g) was found to be higher than in carrot, spinach, mustard, green onion, garland chrysanthemum, sweet potato, green pepper, yellow corn, lettuce, cabbage and celeriac[15]. Research studies such as the ones mentioned suggest basil to be nutritious and thereby likely to be beneficial for human health. Hence, basil's use and popularity have increased over time[16] and account for 40% of the total herb production at the UK's largest herb grower – Vitacress.

Basil is sensitive to chilling temperatures and upon treatment with chilling temperatures shows strong wilting and discoloration of the leaves leading the herb to have a shorter shelf life than when treated at ambient temperatures[17]. There are, however, other herbs that tolerate, and prefer, being transported at chilling temperatures, as the conditions minimise the herbs' contamination with microorganisms and tend to increase their shelf life. Therefore, to avoid chilling damage of basil, all of the herbs, including basil, are transported at ambient temperatures (12-15^{o}C). Although, these temperature are suitable

for basil, they are not ideal for other herbs that prefer being transported at lower temperatures for maximum shelf life.

Improved chilling tolerance in basil would allow all herbs to be transported at lower temperatures without fear of damage occurring and thus maximise shelf life and minimise wastage. The aim of the project, therefore, was to understand the molecular basis of the perception of cold and increase basil's tolerance towards chilling temperatures through changes in the growing conditions.

1.2 Chilling sensitivity

There are 350,000 species of plants on Earth, and they all vary significantly in their responses to chilling temperatures[18].While plants cultivated in tropical and sub-tropical temperatures have failed to acquire the ability to tolerate chilling temperatures in the course of evolution, temperate plants, on the other hand, can survive and flourish even at freezing temperatures[18]. Plants that show damage at temperatures between 0-12^{0}C are referred to as chilling sensitive while plants that show injury at temperatures below 0 ^{0}C are freezing sensitive[19,20]. In this thesis both the terms chilling and cold refer to the temperatures between 0-12^{0}C and are used interchangeably.

According to Sanghera *et al.*[21] chilling temperature leads to a loss of approximately $2 billion in crop production per year worldwide. Chilling temperature not only reduces the crop's quality but also reduces the possibility of crop cultivation and use in other more temperate regions. There are numerous physiological and molecular changes occurring in the plant that either play a role in perception, signalling or in response to chilling temperature stress.

1.3 Perception of chilling temperature stress

Plants are sessile organisms and incapable of escaping from the changing environment. Therefore, they have developed mechanisms that enable them to endure challenging environmental conditions that may threaten their survival and reproduction [22]. However, there are various environmental conditions that may be unfavourable for plant growth and development that include drought stress, nutrient stress, salt stress, pathogen attack and temperature stress[23]. These different stressors elicit responses that either cross–talk or are specific to different stressors, therefore, to elicit responses that are suitable and specific to these different types of stresses, the plant must first be able to perceive and identify the environmental condition that it is exposed to [22]. This, in turn, will allow appropriate signalling to occur and thereby elicit a suitable response to the stressor[24]. Therefore, an understanding of changes that occur in plants and contribute to the perception of the environmental condition is important, as these are the critical steps in determining the survival of the plant at times of stress[24]. Understanding perception in plants may also be important because, in order to improve the crop's yield and quality at times of unfavourable environmental conditions, scientists can consider targeting the changes at the perception phase to improve the plant's tolerance towards these stressors. Hence, in this chapter, the following sections focus on the changes that occur at times of low temperature and the mechanisms that allow the plant to detect or perceive the temperature drop in the environment. Throughout this chapter, low temperature refers to temperatures below 10^0C but above freezing temperatures ($\leq 0^0$C).

1.3.1 Role of cell membrane in the perception of chilling temperature:

1.3.1.1 The lipid composition of the cell membrane's affects the membrane's fluidity at chilling temperature:

Upon exposure to temperatures below 10^0C the damage observed on the shoots of the chilling sensitive plants is believed to commence from the plant cell membrane. Each cell expends approximately 5% of its genes to generate 1000 different lipid species (lipidome). Much of this lipidome is used for the formation of the membrane for the cell and its organelles[25].

The three major classes of lipids most commonly found in membranes include phospholipids, glycolipids and sterols[26]. The structure and size of the hydrocarbon chains of the phospholipids are important as they can affect the fluidity of the membrane[27] (Figure 1.1). The two different classes of phospholipids that are most commonly found include glycerophospholipids (phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), phosphatidylinositol (PtdIns) and phosphatidic acid (PA)) and sphingolipids.

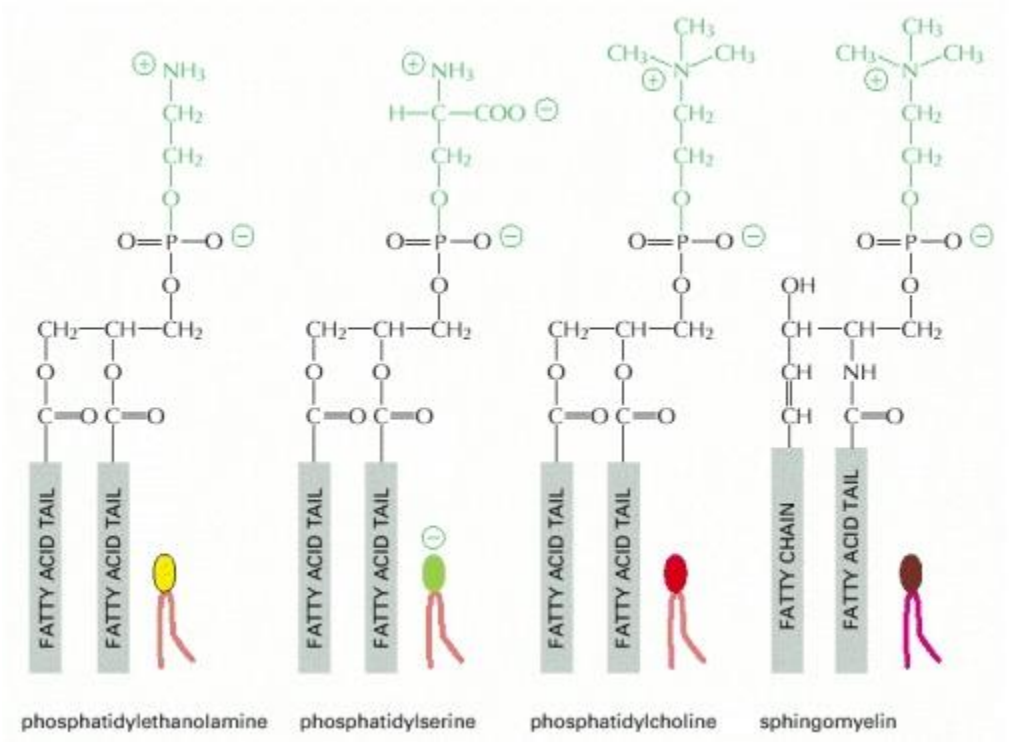


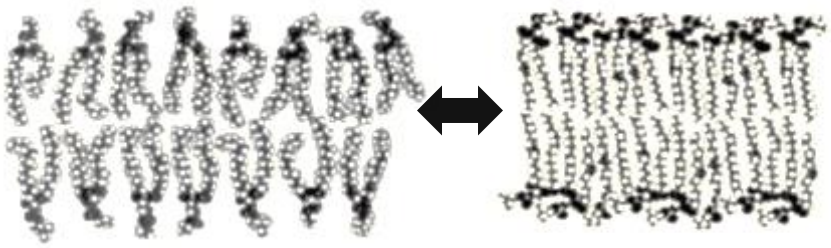
Figure 1.1 Structure of four different types of glycerophospholipids that include phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine and sphingomyelin from left to right, respectively. Reproduced from Alberts *et al.*[27].

Glycerophospholipids consist of cis-unsaturated acyl chains that maintain the membrane's phase fluid at room temperature[25] (Figure 1.1). Sphingolipids consist of saturated hydrocarbon chain or trans unsaturated acyl chain, and so are able to pack more tightly than glycerophospholipids resulting in the adoption of solid or gel phase[25].

There are mainly three different phases that biomembranes can exist in, and that includes: liquid disordered (liquid crystalline), liquid ordered (raft) and solid gel phase[25]. Lipid composition of the membrane such as the type of phospholipids and the content of sterols can determine the phase of the cell membrane at physiological temperatures. Glycerophospholipid-enriched membranes adopt a liquid disordered phase whereas sphingolipid-enriched membranes may form a solid gel phase[25]. Sterols are nonpolar lipids of the cell membrane and do not form bilayers. Instead, they preferentially interact with sphingolipids and fluidize them resulting in the adoption of the liquid ordered phase[28]. This phase is largely solid but can allow translational mobility of the lipid molecules within the

membrane equivalent to that of a liquid phase. The plasma membrane is enriched with sphingolipids and sterols to a greater extent than glycerophospholipids[25];this renders the membrane sturdy to provide mechanical strength and support for the cell but also flexible to allow easy diffusion of the lipid molecules within the membrane. The three different lipid species of the lipidome are completely miscible and render the plasma membrane on an overall liquid disordered phase at room temperature.

The type of phase adopted by the biomembrane at ambient temperatures is largely dependent upon its lipid composition. At low temperatures, however, the lipids of the membrane may undergo a reversible change of state, from fluid (disordered) to a non-fluid (ordered) state that is also referred to as liquid phase and solid gel phase, respectively[29,30] (Figure 1.2).



Ambient temperature Cold temperature

Figure 1.2 Cell membrane at a physiological temperature in a liquid disordered phase (left) reversibly shifted to sold-gel ordered phase at low temperature (right). Reproduced from Meer *et al.*[25] (2008).

The change of the membrane phase is likely to take place faster in a membrane with a high composition of saturated fatty acids (sphingolipids)[30]. The sphingolipids acyl chains pack closely together and have greater van der Waals forces. If the temperature decreases, the van der Waals forces are increased making the membrane more rigid and restricting the mobility of the lipids within the membrane[31]. The restricted mobility of the lipids disrupts its interaction with the membrane embedded proteins. The proteins adopt an irreversible conformational change that either makes them denatured or more susceptible to proteolysis. The double bond in the acyl chain introduces a 30^0 bend that elbows the

neighbouring acyl chain and resists them from packing closely together. The increased surface area occupied by unsaturated fatty acids reduces the van der Waals forces between acyl chains[31]. Hence, a high proportion of unsaturated fatty acids in the membrane increases fluidity and delays the transition from liquid crystalline to a solid gel phase.

1.3.1.2 Membrane rigidification is a primary sensor of chilling stress.

At ambient temperatures, the fluid state of the membrane is defined as the extent of molecular motion and disorder in phospholipids of the lipid bilayer[32]. Infrared spectroscopy (IR) measures the frequency of the molecule vibrations when irradiated with infrared light[33]**.** Upon irradiation by infrared light, the chemically bonded atoms of the molecule reach an excitation level and vibrate either by stretching (length of the chemical bond changes) or bending (angle of the chemical bond changes) [34]. The structure of the molecule can only absorb a certain number of photons with particular energy. Hence, each molecule would produce a vibrational spectrum that is unique and is a characteristic of the molecule [35]. Thus, the infrared spectrum of molecules can be used as a fingerprint to find the identity of unknown molecules[35]. Similarly, spectra of phospholipid molecules can be obtained at two different temperatures to identify whether the frequency vibrations of the molecules are temperature dependent.

Fourier transform infrared spectrometry (FTIR) is an advanced technique of infrared spectroscopy with a greater sensitivity and can provide information on the stretching vibration on all regions of the phospholipid molecule, simultaneously [36]. FTIR is widely used to assess changes in the vibrations of the acyl chain of phospholipid molecules at different temperatures [37]. The stretching vibration frequency of the acyl chain decreased by 2-5 cm^{-1} near the bandwidth range of 2851 cm^{-1} (the strongest absorption band for C-H bonds) on the transition of the membrane from liquid to gel-like state [38]. The isolated cytoplasmic

and thylakoid membrane's phospholipid molecules also showed reduced stretching vibration frequency at temperatures lower than the growth temperatures[39]; proving that the mobility of the molecules decreases at chilling temperature.

Plants can tolerate freezing temperatures by acclimating to cold temperatures[40]. Cold acclimation in Alfalfa plants is achieved by increased expression of cold-responsive and acclimation-specific genes (*cas30*$^{+}$) that are in turn induced by a temporary increase in the cytosolic Ca^{2+} ion concentration [41]. Orvar *et al*.[41] used cold acclimation markers (*cas30* mRNA and cytosol Ca^{2+} concentration), membrane rigidifiers DMSO (dimethyl sulfoxide) and fluidizers BA (benzyl alcohol) to elucidate the role of membrane rigidification in activating downstream events involved in the cold acclimation response of Alfalfa plant cells. DMSO when added to the Alfalfa cell suspension at a concentration of 1% and caused an increase in the cytosolic Ca^{2+} concentration that led to an increase in mRNA transcript levels of *cas30* at 25^0C. This response of Alfalfa cell suspension was reversed when BA was added at 4^0C. The findings of the study clearly showed cell membrane rigidification to occur prior to the temporary increase in Ca^{2+} concentration (Figure 1.3) and demonstrated the possible importance of membrane rigidification in activation of downstream events normally involved in the cold acclimation process of Alfalfa plant cells.

FADS are desaturases that convert a single bond between two carbon atoms into a double bond in the acyl chain of membrane glycerophospholipid molecules, mostly in the cis configuration and thereby convert a saturated hydrocarbon chain into an unsaturated one[42]. Using three different genotypes of *A. thaliana* cell suspension, Vaultier *et al*.[43] carried out a study where a correlation between the accumulation of the cold signalling molecule phosphatidic acid (PA) and membrane rigidification was observed. In the study, FAD2 mutant, wild-type and FAD3+ (overexpressed) genotypes were subjected to a low temperature from 22^0C to 3^0C for one minute. The accumulation of PA in FAD2 mutant, wild-type and FAD3+ was detected at 18^0C, 14^0C and 12^0C, respectively. The order of PA accumulation correlates with the extent of membrane rigidification in those genotypes.

Hence, the study by Vaultier *et al.*[43] adds support to the idea of membrane rigidification of cells occurring upstream to the production of cold signalling molecules such as PA in higher plants.

1.3.2 Reduced enzymatic activity leads to reduced rate of metabolism

Metabolism is a series of reactions catalysed by enzymes that take place sequentially to maintain the living state of the cell. The rate of these reactions depends on enzyme activity that reduces with temperature (Figure 1.3). In the process of photosynthesis, CO_2 combines with ribulose-1,5-bisphosphate (RuBP) in the stroma of the chloroplast to generate glycerate-3-phosphate that is then reduced to triose-phosphate (TP) [44]. The TP enters either into the starch synthesis pathway in the chloroplast or the sucrose synthesis pathway in the cytosol; TP is then converted into sucrose, and an inorganic phosphate (Pi) is released that is transported from the cytosol to the chloroplast to regenerate RuBP [45]. When exposed to chilling temperatures, all of the metabolic reactions including sucrose synthesis are reduced [46], leading to the reduced release of Pi into the cytosol (Figure 1.3). This results in reduced exchange of TP for Pi, leading to reduced regeneration of RuBP for photosynthesis[47]. Low levels of Pi trigger the expression of genes coding for sucrose synthesis enzymes, chilling temperature responsive genes and also increase production of reactive oxygen species (ROS) [44,48] (Figure 1.3). Reduced regeneration of RuBP causes an imbalance between the amount of light energy absorbed and its subsequent use by metabolism, in turn this leads to an increase in the production of ROS [49]. ROS are free radical molecules such as superoxide anions, hydroxyl radicals and hydrogen peroxide and are produced by the plant as part of normal metabolism. However, at chilling temperature, the activity of the ROS scavenging enzymes is lowered and are therefore not able to counterbalance the increased formation of ROS [49].

In Japonica rice, 60% of the cold-responsive genes were induced by increased levels of ROS[50] and demonstrated the contribution of ROS in cold acclimation of Japonica rice. These examples suggest that low Pi content in the plant may play an important role in signalling by causing increased production of ROS that in turn can also contribute to plants' cold response (Figure 1.3).

It is the reduced enzymatic activity at the time of chilling temperature that causes metabolism to slow down that in turn reduces the availability of cytosol Pi, increases production of ROS, and induces cold-responsive gene expression (Figure 1.3). Hence, reduced enzymatic activity is considered to be a part of temperature perception machinery.

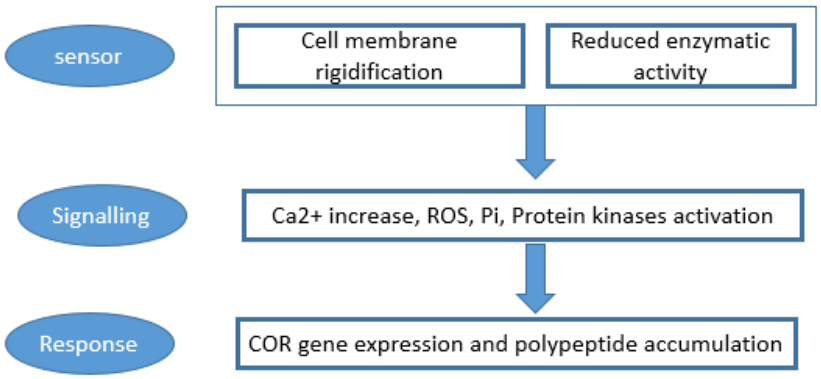


Figure 1.3 The sensing and signalling machinery initiating responses that together make up a cellular response to chilling temperatures. Reproduced and modified from Ruelland and Zachowski[44].

In addition, although the changes occurring within the cell and to its constituents are well known, the detection speed of the temperature change perception may vary from one sensor to another. For instance, it may take a few seconds for membrane fluidity to decrease at chilling temperatures but may take few minutes for other organelles to respond to temperature changes[51]. Furthermore, although membrane fluidity reduction and enzymatic activity reduction have been well identified as sensors and supported with evidence coming from mutant studies, there may still be more sensors remaining for

scientists to discover. For example, Kumar & Wigge[52] have recently found chromatin remodelling to occur at times of temperature change in the environment. At ambient temperatures, the chromatin is loosely packed allowing access to the binding sites for a transcription factor to initiate genes expression. However, at times of chilling temperature, the chromatin is more tightly wrapped around the histone proteins H2A.Z preventing access to sites for transcription factors and reducing expression of certain genes. According to Knight and Knight[53], chromatin folding at low temperature is an attractive hypothesis as it provides a direct link between chilling temperature and change in expression of genes in response to that temperature change. However, the stage at which chromatin remodelling takes place is still not yet known or if it forms a part of perception or signalling. Therefore chromatin remodelling is a target for future research, and more evidence is required to elucidate its role in the response of the plant when exposed to chilling temperatures. Furthermore, plants can detect changes in temperature by as little as 1^0C, but these changes can happen at any time of the day or during any season[53]. Therefore, there is a possibility that sensors may have a threshold of the drop in temperature to which they may respond. On the other hand, the plant may require more than one sensor to be switched on, and for a specific period, before initiating a cold response. Research on chromatin remodelling suggests that there is no one thermometer existing in the plant [44] but rather several, and these may not only signal the plant of the temperature change but also differentiate between temperature changes that are worthy of a response and those that are not. Hence, there are these remaining questions that have not yet been answered and therefore remain a target of future research.

1.4 Signalling of chilling temperature

Cold acclimation in the plants requires activation or repression of various cold-responsive genes. Therefore, the perception of a drop in the temperature has to be transmitted from

the cell membrane to the nucleus[54]. Cold signalling to the nucleus could be achieved via increased concentration of cytosol signalling molecules such as Ca^{2+} and ROS that cause activation of Mitogen-activated protein kinase MAPK pathways and other protein kinase cascades.

1.4.1 Cytoskeleton dissembles at times of chilling temperature leading to the influx of Ca^{2+} ions from the extracellular environment to the intracellular:

The cytoskeleton is a network of protein filaments including actin filaments and microtubules. These filaments extend throughout the cytosol and are involved in maintaining the cell shape and function. Following cell membrane rigidification, the cytoskeleton was found to disassemble at times of chilling temperature stress[55]. The disassembly of microfilaments has been suggested to occur upstream to calcium influx in the cell[53]. Whether this change in the cytoskeleton disassembly was in response to a change in the temperature of the environment was investigated by Orvar *et al.*[41]. It was found from *Medicago sativa* cells that the accumulation of Ca^{2+} ions and induction of *Cas30* was prevented by application of jasplakinolide (cytoskeleton stabiliser) at 4^0C. However, on the application of cytochalasin D (cytoskeleton destabiliser), there was increased accumulation of *Cas30* mRNA transcript and intracellular concentration of Ca^{2+} ions at 25^0C [44]. The findings of the study by Orvar *et al*[41] prove that changes in cytoskeleton assembly occur upstream to Ca^{2+} and *cas30* mRNA induced expression. Due to the continuum of the cytoskeleton and the cell membrane, it has been suggested that changes in the physical state of the membrane may cause a change in the cytoskeleton assembly[44]. However, the mechanism of signalling that transmits information from the membrane to the cytoskeleton is still not yet known.

According to Orvar *et al.* [41], the cytoskeleton is directly or indirectly connected to calcium channels. The intact cytoskeleton in the cytosol creates tension that keeps the calcium channels closed (Figure 1.4). Therefore during chilling temperatures, the cytoskeleton

disassembly results in alleviation of the tension and calcium channels open (Figure 1.4). The Ca^{2+} ions from extracellular spaces then flow into the cell, increasing intracellular cytosol Ca^{2+} concentration (Figure 1.4). It was proposed by Ruelland *et al.*[56] that increased Ca^{2+} may activate phospholipase C (PLC) and phospholipase D (PLD) through trimeric G proteins and this was proposed due to the activation of PLD in barley being mediated by multimeric G protein. The endoplasmic reticulum is the major store of Ca^{2+} ions inside the cell and contains Ca^{2+} pumps that cause accumulation of Ca^{2+} ions in the endoplasmic reticulum (Figure 1.4). The lumen of the endoplasmic reticulum is continuous with the perinuclear space lumen of the nuclear envelope, and therefore Ca^{2+} accumulated in the endoplasmic reticulum can diffuse freely into the lumen of the nuclear envelope [32]. Activation of PLC and PLD leads to the production of IP_3 that binds to the receptors on the endoplasmic reticulum and nuclear membrane and causes Ca^{2+} ions to be released from its intracellular stores into either the cytosol or the nucleoplasm, respectively [57] (Figure 1.4). According to Mauger[57], the increase in nuclear calcium concentration is initiated by the release of Ca^{2+} ions from the nuclear envelope but can be propagated by free diffusion of Ca^{2+} ions from the cytosol into the nucleoplasm through the nuclear pore complex. Ca^{2+} in the nucleus can bind to proteins such as calmodulin (CaM), calcium-dependent protein kinase (CDPK) and phosphatases in the nucleus to influence the activity of transcription factors either at the transcriptional or post-translational stage and thereby affect the expression of their target genes (Figure 1.4)[58].

Monroy *et al.*[59] exposed alfalfa cell suspension to 4^0C for eight days and found them to achieve cold acclimation successfully, but when the Ca^{2+} chelators that sequester Ca^{2+} ions extracellularly were added to the cell suspension, the cold acclimation declined by 70%. On addition of La^{3+} and verapamil, blockers of cell membrane's Ca^{2+} channels, the development of freezing tolerance was completely abolished. These findings suggest that Ca^{2+} ions concentration inside the cell play an important role in the development of cold tolerance in alfalfa cells.

In plants, a variety of biotic and abiotic stresses, such as pathogens, cold, drought, and salt stress, cause a transient rise in intracellular Ca^{2+} concentration[60]. There is a generic Ca^{2+} signalling mechanism that takes place in plants in response to abiotic stimuli, but in order to survive, the plant must respond specifically, and appropriately, to the stressor[61]. Therefore, the stressor induces a transient Ca^{2+} rise in intracellular concentration with specific characteristics (phases, duration and magnitude of cytosol Ca^{2+} concentration) that encode specific responses in plants against the stressor[60]. These small but crucial differences in stress-induced Ca^{2+} elevation are termed calcium signatures[60]. It is the decoding of these calcium signatures that determines the overall response of the cell.

Ca^{2+} signatures are translated by calcium sensors such as CaM, CDPK, Calcineurin B-like (CBL) and CBL interacting protein kinase (CIPK)[24]. Ca^{2+} activates CaM and in turn, CaM regulates the activity of transcription factors such as CAMTA3, which in turn regulates its target gene expression. CDPK, when activated, phosphorylates many regulatory proteins including transcriptional factors which then regulate the expression of their target genes[62]. N-(6-amino-hexyl)-5-chloro-I-napthalene-sulfonamide hydrochloride, an inhibitor of CDPK and CaM, was found to inhibit cold acclimation completely in alfalfa protoplasts, whereas an inhibitor of protein kinase C reduced cold acclimation by 50%[59]. This shows the importance of Ca^{2+} signature translation by its sensors in the development of cells' cold tolerance. CBL proteins need CIPK to influence the activity of transcription factors. CBL1 interacts with CIPK7 and the complex binds onto transcription factors to affect the expression of their target genes that regulate specific biochemical processes and protect plants from chilling injury[63]. One of the cold regulatory pathways that is activated by elevated Ca^{2+} concentration is the C-repeat binding factor (CBF) pathway[24]. The CBF pathway is one of the most important cold regulatory pathways that is well known to impart chilling temperature tolerance in a variety of different plant species, including plants that are tolerant and sensitive to chilling temperatures.

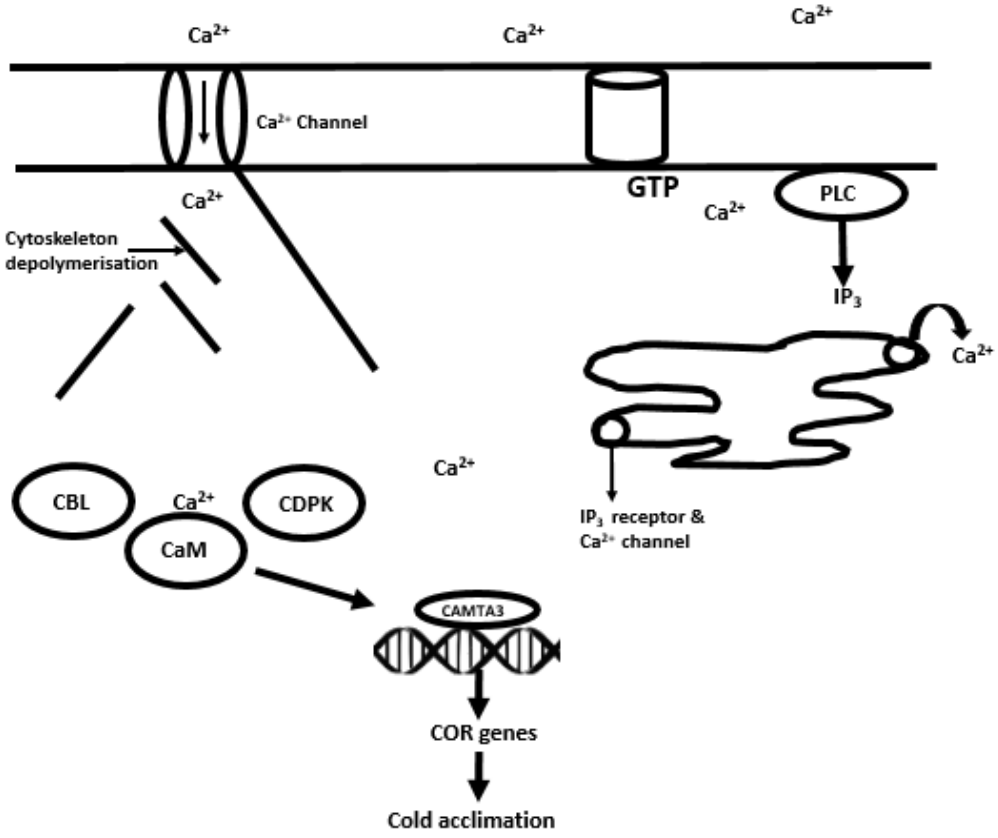


Figure 1.4 Schematic demonstration of calcium signalling occurring after membrane rigidification and cytoskeleton destabilisation at times of low temperature resulting in induction of cold responsive genes and cold acclimation in plant cells.

1.4.1.1 The CBF pathway

The CBF1, CBF2 and CBF3 genes are located on chromosome IV in *A. thaliana*[64]. The CBF locus consists of three genes arranged in a tandem array encoding transcription factors CBF1, CBF2 and CBF3, also known as dehydration responsive element (DREB1B, DREB1C, and DREB1A), respectively[65]. These transcription factors are members of the AP2 family of DNA binding proteins and, when expressed, they bind to the CRT/DRE element in the promoter regions of their target genes, also referred to as the CBF regulon, to initiate their expression. It is this induction of CBF regulons that cause plants to develop tolerance towards chilling temperatures. In *A. thaliana,* 12% of the genes responsive to chilling temperature were accounted by the CBF pathway that makes up to 4% of the total number

of *A. thaliana* genes[66,67]. This represents the importance of CBF pathways in plants response to chilling temperatures. Some economically important crops have been genetically engineered by targeting the CBF pathway to induce their tolerance towards chilling temperatures. For instance, CBF2 and CBF3 were overexpressed in rice leading to increased cold tolerance in transgenic rice[67]. However, constitutive expression of CBF genes comes at the cost of growth retardation, but this limitation can be overcome by using CBF genes from a different source[67]. For instance, CBF gene HvCBF4 expressed from barley in rice resulted in increased cold tolerance without growth retardation[68].

1.4.1.2 Regulation of CBF pathway at ambient temperatures by the circadian clock:

Living organisms (plants, animals, fungi and insects) possess the ability to anticipate the arrival of dawn and dusk and adjust their physiological and biochemical processes to the local time of the environment[69]. Programming of metabolism to the conditions of the environment is advantageous to the organisms as it allows the biological process to occur at the time of the day that it is likely to be of the most benefit. For instance, *A. thaliana* plants with an entrained biological rhythm to that of the local time fixed more carbon, grew faster and survived better than plants whose biology was not synchronous with the environment[70]. The endogenous timekeeping mechanisms that create and maintain oscillation of organism's biology over a period of 24 h are referred to as the circadian clock. However, for this entrainment to be beneficial, the biological rhythm of the organism must be in a match with that of the environment[70]. Hence, the circadian clock is reset each day by the two external stimuli such as light and temperature that are perceived by the plants at dawn and dusk[69]. These light signals are transmitted to the circadian clock by the light absorbing photoreceptors[71]. There are mainly three different types of photoreceptors that absorb different wavelengths of light from the visible and UV range of the electromagnetic spectrum[72]. Phytochromes (PHYA-PHYE) exist in two forms that include an active red light absorbing (Pfr) and an inactive far red light absorbing (Pr) form[69]. Cryptochromes and

phototropins, on the other hand, absorb blue and UV-A light[72]. Phytochromes and cryptochromes account for all of the de-etiolation in plants as well as regulation of clock components that entrain the plants rhythm to the local time of the enviroment[73].

The circadian clock consists of a complex negative feedback loop. The transcription factors, circadian clock-associated 1 (CCA1), and late elongated hypocotyl (LHY) are expressed at dawn and negatively regulate the evening complex (EC) consisting of early flowering 3 (ELF3), early flowering 4 (ELF4) and Lux arrhythmo (LUX). The EC, in turn, represses a group of daytime-phased transcription factors, the timing of cab expression 1 (TOC1, also called pseudo response regulator 1, PRR1), and PRR5, 7 and 9. Ultimately, TOC1, PRR 5, 7 and nine feedback to repress CCA1 and LHY; therefore, by the end of the day, CCA1 and LHY levels are reduced significantly[74].

The first light of the day is termed Zeitgeber time (ZT) 0. CCA1 and LHY protein levels peak after dawn (ZT1-ZT3)[65]. At this time, CCA1 and LHY bind on to the evening-element (EE) of the promoters of the EC components and repress its expression. However, CCA1 and LHY also promote expression of PRR7 and PRR9 via the CCA1 binding site (CBS) in their promoters. CCA1 and LHY also bind on the promoters of CBF transcription factors and induce their expression[75]. The CBF's, PRR7 and PRR9 peak similarly at ZT8[65]. The CBF's then induce expression of their CBF regulon whose expression then peaks at ZT10.

During the day, and at ambient temperatures, the CBF is regulated positively by CCA1 and LHY, CBF peak at ZT8 and trough at ZT20[67]. However, in the evening the CBF is negatively regulated by the circadian clock when the CCA1 and LHY levels drop, and there is no stimulation occurring of the CBF pathway. There is repression instead being exerted from Phytochrome interacting factor (PIF7). PIF7 physically interacts with TOC1 and forms a PIF7-TOC1 protein complex that then binds on to the G-box element in the promoters of CBF genes and in turn, suppresses their expression[76].

1.4.1.3 Regulation of CBF pathway at chilling temperatures:

At chilling temperatures, the CBF pathway is also positively regulated by two transcription factors; INDUCER OF CBF EXPRESSION 1 (ICE1) and CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 3 (CAMTA3) (Figure 1.5 and 1.6). ICE1 and CAMTA3 bind on the promoters of CBF genes and upregulate their expression, which in turn leads to induction of cold-responsive genes (COR) genes and thereby provides tolerance to the plants against injurious chilling temperatures. ICE1 and CAMTA3 are encoded by genes that are not transcriptionally regulated, but their transcriptional activity is affected by post-translational modifications. HOS1 is an E3 ubiquitin ligase that causes ubiquitination of ICE1 and leads to its proteasomal degradation[77] (Figure 1.5). SIZ1 and SIZ2 are conjugating SUMO enzymes that catalyse covalent attachment of SUMO proteins with their target protein to prevent the target proteins from proteasomal degradation, thereby stabilising them[78] (Figure 1.5). Chaikam and Karlson[79] proposed a hypothetical model regarding ICE1 regulation at a cold temperatures that involved three components: HOS1, SIZ1, and ICE1. It was suggested that under normal conditions HOS1 would target ICE1 for degradation by the proteasome. However, at chilling temperatures, the SIZ1 would be activated, and HOS1 expression would be inhibited. This would dominate the stability of ICE1 over degradation at chilling temperatures that would, in turn, result in the greater induction of the CBF pathway at chilling temperatures. However, although greater levels of ICE1 leads to greater expression of CBF genes, a report on the overexpression of ICE1 gene in *A. thaliana* at ambient temperature showed that there was no induction of the CBF3 gene or its regulons [80]. This indicates that an additional post-translational modification must take place at chilling temperatures that influence the activity of ICE1, rather than its amount, that results in the proper induction of CBF genes.

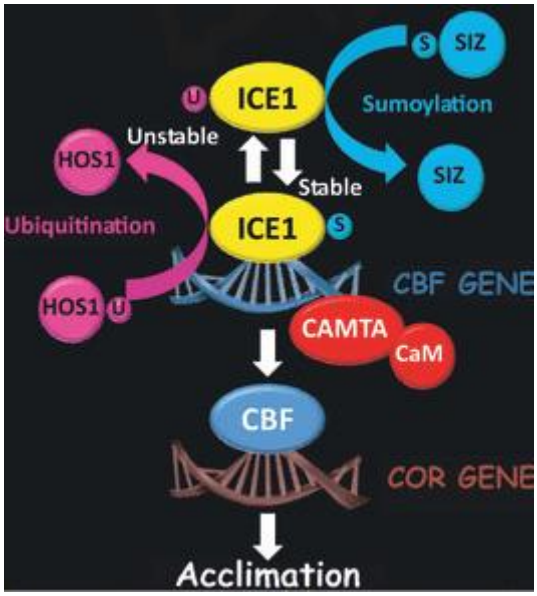


Figure 1.5 The regulation of the transcription factors involved in the promotion of CBF gene expression. Reproduced from Knight and Knight[20].

Ding *et al.*[77], through screening of protein kinase mutants of *A. thaliana*, found that a mutation in open stomata 1 (OST1) resulted in reduced induction of CBF regulon by ICE1. Ding *et al.*[77] also showed that OST1 is rapidly induced in its protein kinase activity at chilling temperatures and phosphorylates ICE1 which in turn increases ICE1 function in activating CBF gene expression in cold conditions in *A. thaliana*. Overexpression of OST1 was also found to induce CBF regulon expression more strongly and led to greater freezing tolerance than seen in wild-type plants [77]. However, the low temperature receptor that leads to activation of OST1 is still not yet fully known but it has been hypothesised that it may be the increased cytosol Ca^{2+} concentration resulting from Ca^{2+} influx at the time of cytoskeleton disassembly that may lead to activation of OST1. However, evidence to support this predicted link between elevated cytosolic Ca^{2+} concentrations leading to activation of OST1 phosphorylation activity is still lacking and is, therefore, a target of future research.

Increased Ca^{2+} concentration results in binding of the Ca^{2+} to the calmodulin protein and thereby activating it. Activated calmodulin may interact with CAMTA3, and cause modification in DNA binding activity of CAMTA3. CAMTA3 is then able to bind and promote

expression of the CBF2 gene and its regulon resulting in increased tolerance of the plant to chilling temperatures[81] (Figure 1.6). These findings show the importance of calcium's role in signalling of the chilling temperature to induce a response appropriate for protection against such injurious environmental conditions. It is also evident that even though ICE1 and CAMTA3 genes are expressed at ambient temperatures, their role in cold signalling does not become active until there is an actual decrease in the temperature of the environment.

At chilling temperatures, during the day CCA1/LHY and ICE1/CAMTA3 work synergistically on CBF gene promoters to maximise the upregulation of CBF pathway (Figure 1.6). However, during evening hours the CCA1 and LHY levels are low therefore upregulation of CBF pathway is only mediated by ICE1 & CAMTA3 (Figure 1.6). Hence, the magnitude of CBF pathway's upregulation is reduced in evening hours.

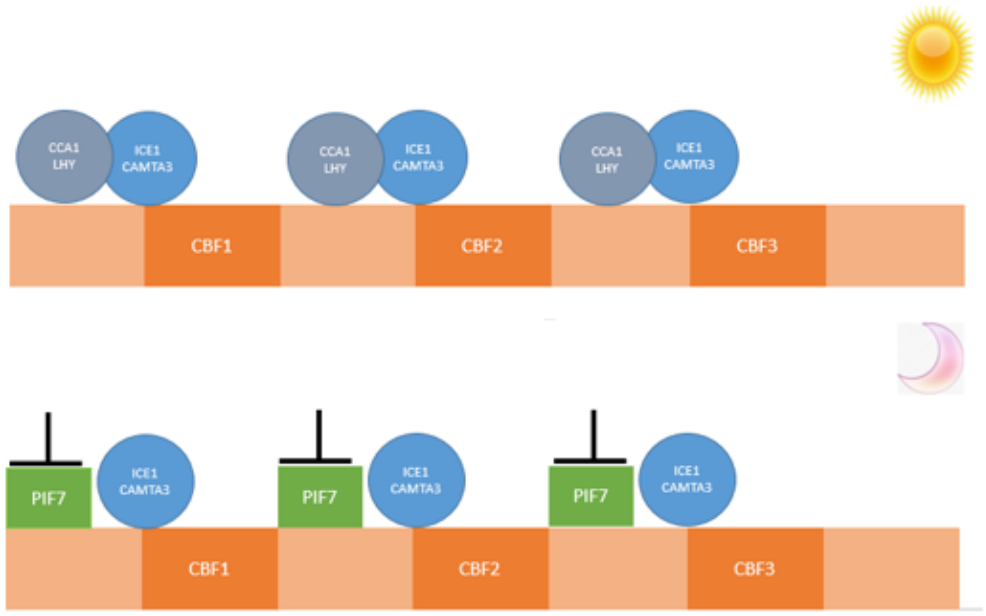


Figure 1.6, The regulation of CBF pathway by the circadian clock and low temperature. Reproduced and modified from Dong *et al.* (2011)[65] During the day when the plant is transferred to low temperature, CCA1/LHY and ICE1 & CAMTA3 act synergistically to stimulate CBF expression that causes the CBF transcripts to be expressed in much higher amounts at chilling temperatures than at ambient temperatures. In the evening, however, while there is stimulation from ICE1 and CAMTA3 genes, the CBF genes are no longer positively regulated by the circadian clock. Instead, CBF genes are downregulated by PIF7 transcription factors acting together with TOC1 at night. Hence, the induction of CBF genes is much lower in the evening than during the day but higher at low temperatures than had the plant been at ambient temperatures.

1.5 The detrimental effects and response of plants to chilling temperatures:

Change in expression of the cold-responsive genes that were induced by signalling of either Ca^{2+}, ROS or CBF's, contributes to the response of plants to chilling temperatures [22]. The COR genes expressed in response to induction by the CBF pathway are known to encode polypeptides that play a role in protecting cellular components against damage by chilling temperatures. The CBF pathway has been suggested to be conserved among various plant species including barley, rye, wheat, tomato, maize[82,83] and rice but the size of the CBF regulon was indicated to vary among different plant species. For instance, CBF regulon size was found smaller in chilling intolerant plant species such as rice and tomato (~10 genes)[84,85] when compared to a chilling tolerant plant *A. thaliana* (~ 85 genes)[86].

According to Beck *et al*. (2004)[20], the detrimental effect of chilling temperatures is a syndrome rather than a symptom; it is where destruction dominates over synthesis. Chilling sensitive plants, when exposed to chilling temperatures, suffer from discolouration of leaves, wilting, delay in ripening of fruits, accelerated ageing, reduced growth and increased susceptibility to decay[87]. The extent of these damaging effects exhibited by chilling sensitive plants varies greatly depending on the duration and the extent of decrease in the temperature and sensitivity of the plant to chilling temperatures[67].

1.5.1 Injuries experienced by chilling sensitive plants on exposure to chilling temperatures:

Intolerant chilling sensitive plants when exposed to chilling temperatures, have a large number of changes taking place at both the physiological and molecular level which result in the manifestation of injury symptoms occurring in the plant tissues. These injuries are described at the cellular, biological processes and at the molecular level in the following sections.

1.5.1.1 Cellular injury caused by chilling temperatures

Membranes are the site of perception of low temperatures and are also the primary sites to experience damage by chilling temperatures. The phase transition from fluid to rigidification depends on several factors including lipid composition, the desaturation of fatty acids and the length of the fatty acid chain[88]. Longer fatty acid chains have greater van der Waals forces and increased surface area for molecules to interact with each other. This results in the formation of stronger interactions between molecules and the long fatty acid chains are therefore more susceptible to rigidification than shorter fatty acid chains. In addition, the greater the proportion of saturated fatty acids, as opposed to desaturated fatty acids, the greater the susceptibility of membranes to chilling temperature, allowing easy transition of phase from liquid to that of solid. It is therefore not surprising that chilling resistant plants from temperate regions tend to have greater desaturated fatty acid composition than chilling sensitive plants [89].

Other cellular changes that have also been observed during chilling stress in some plant species include vascularization of the endoplasmic reticulum in tomato [90], destruction of the photosystems and thylakoid membranes in the chloroplast in cherimoya[91] and reduced number of cristae in the mitochondrion in maize seedlings[92]. These can result in reduced rate of photosynthesis, ATP production and accumulation of toxic substances resulting in the development of chilling injury symptoms in the tissues of the chilling sensitive plants.

1.5.2 Difference in the chilling and freezing induced cellular injury causing activation of different responses for protection and increased tolerance to chilling and freezing temperatures.

Membrane damage is experienced at both chilling and freezing temperatures, however, freezing induced injury is different to the one induced by chilling temperature due to the formation of ice crystal in freezing sensitive plant tissues[93]. For instance, chilling temperature causes rigidification and increased permeability of the membrane while freezing temperature causes protoplast shrinkage due to freezing induced dehydration[94]. In addition, formation of ice crystal induces large endocytotic vesicles in protoplasts of non-acclimated winter rye leaves that lyse upon thawing or rehydration of protoplasts[95]. The electron micrographic studies of *A. thaliana*, winter rye and oat non-acclimated protoplasts have shown a change from lamella to hexagonal II phase of the membrane (Figure 1.7)[96–98]. This phase transition at freezing temperatures cases increased leakage of solute from the cells and eventually result in protoplast shrinkage[93]. According to Yamazaki *et al*[93] tolerance to freezing temperatures involves resistance to change in the membrane phase from lamella to hexagonal II phase (Figure 1.7). The incidence of the phase change is related to lipid composition of the membrane. Thus, resistance to membrane phase change requires change in lipid composition. Zheng *et al*[99] studied the changes in the lipid composition of *A. thaliana* and rice at freezing temperatures and chilling temperatures, respectively. *A. thaliana* and rice both showed an increase in phosphatidic acid and phosphatidylinositol after treatment at chilling or freezing temperatures, respectively[99]. *A. thaliana*, however, contained higher level of galactolipids, double bond index and synthesized large head groups of glycerolipids than rice[99]. The study by Zhen *et al*[99] shows the changes in lipid composition needed to increase tolerance at freezing temperatures. Changes such as increased accumulation of antioxidants may

be able to protect membranes and increase chilling tolerance[100]. However, the same changes will not be able to provide protection to non-acclimated protoplasts at freezing temperatures that experience shrinkage in response to dehydration and mechanical stress from freezing temperatures.

Tolerance from mechanical stress on plasma membrane of protoplasts at freezing temperatures involve surface area regulation system and increased Ca^{2+} ion concentration[93]. Surface area regulation is a system that senses changes in the tension of the plasma membrane and responds to them to prevent membrane deformation by either extending or retrieving plasma membrane material[101]. On increase in the tension beyond a set point the membrane extends its material to relax and reduce the tension until it is restored to its original set point. Conversely, the membrane retrieves its material at times of reduced tension in the membrane[101]. Freeze induced vesides in protoplasts form upon formation of ice crystals in plant tissues. These vesicles are part of surface area regulation and reduces deformation of membranes[93]. Hence, increased surface area regulation is associated with increased tolerance to freezing temperatures. However, surface area regulation failed to occur at low Ca^{2+} ion concentration, and, as a result, the recognition of mechanical stress in the membrane may involve the use of a calcium channel[93].

A non-edible *Jatropha curcas* plant used for biodiesel production was exposed to chilling and freezing temperatures to study the impact of these temperatures on the plants photosynthetic apparatus[102]. The study showed that the photosynthesis declined at both the temperatures. However, the decline in photosynthesis was only by 75% at chilling temperatures and 100% at freezing temperatures[102]. In addition, reduced relative water content and chlorophyll content was observed but only at freezing temperatures[102]. The study by Grimaud *et al*[103] identified chloroplast changes unique to tolerance in chilling or freezing temperatures. Chilling tolerance in pea was

found associated to accumulation of proteins with antioxidative and osmoprotectant properties[103]. However, tolerance to freezing temperatures involved increase in the capacity of photosynthesis, stability of photosystems, carbohydrate and protein synthesis.

The studies looking at plants responses to chilling and freezing temperatures show that severe injury is induced by freezing temperatures and therefore require induction of more complex defense system. However, plants that are chilling tolerant such as *A. thaliana* are able to survive freezing temperatures upon acclimation by storage at chilling temperatures. Chilling sensitive plants on the other hand can also show increased tolerance to lower chilling temperatures when also acclimated at sub optimal chilling temperature but may not be able to cope with formation of ice crystals in its plant tissues.

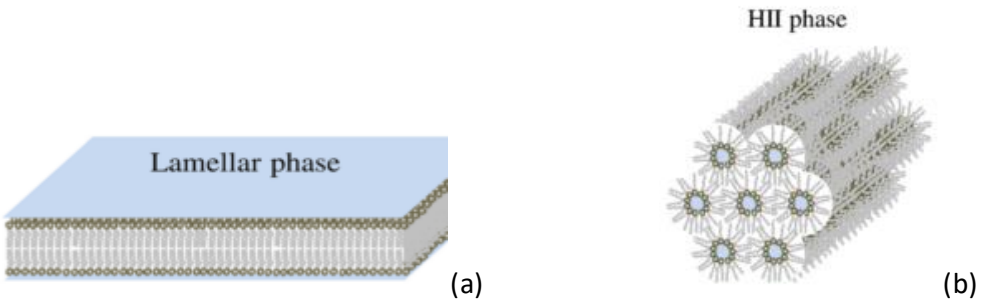


Figure 1.7 The bilayer membrane commonly exists in a planar structure also called lamellar phase (a). Reproduced from Jouhet[104] However, at times of environmental stress the lipids in the membrane can aggregate and form different shapes. At low temperatures, the cell membrane can adopt a hexagonal II phase in a cylindrical shape with the hydrophilic region facing the inside and the hydrophobic region facing the outside of the tube (b). The cylindrical shapes then stack on top and adjacent to each other resulting in the formation of pipes that act as aqueous channels. This form of lipid organisation results in the finite hydrophobic surface to be in contact with

water. However, it is the close packing of the cylindrical shape that keeps the hexagonal II phase of lipids stable.

1.5.2.1 The role of Reactive Oxygen Species (ROS) in plants at times of chilling stress:

Membrane damage could also occur by the actions of ROS which is generated as a result of electron leakage in the electron transport chain of organelles including the chloroplast and mitochondria. The leaked electron from the electron transport chain is accepted by oxygen resulting in the production of free radical oxygen species that includes molecules such as superoxide; hydrogen peroxide; hydroxyl radicals; hydroxyl ions; and nitric oxide [105]. Although ROS are largely produced by the organelles as part of the normal metabolism their generation is also influenced by biotic and abiotic stresses such as ionising radiation, salt stress, drought, heavy metals and temperature extremes[106]. ROS are highly reactive and can either serve as a signalling molecule to regulate the physiological and biological processes to maintain the function of the cell or as an oxidant where they react with lipids, proteins, DNA and carbohydrates, creating oxidative stress[107]. According to Schieber & Chandel [108], there is an increased level of ROS-activated genes and signal transduction pathways to adapt the cell to the changing environment. At times of abiotic stress, the elevated levels of ROS could cause deleterious effects on the cell. Hence, the cell produces enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) and non-enzymatic antioxidants such as Ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, and phenolics, to stabilize the ROS by donating an extra electron and thereby preventing the ROS from reacting non-specifically with crucial molecules within the cell[106]. Whether ROS will act as a signalling or damaging molecule depends on the delicate equilibrium between ROS and antioxidants. At times of abiotic stress, the equilibrium between antioxidants and ROS is disturbed leading to a sudden increase in ROS, and this

results in the generation of oxidative stress in cells [105]. This oxidative stress can pose a threat to a cell by either causing peroxidation of lipids, oxidation of proteins, and damage to nucleic acids, enzyme inhibition, activation of the programmed cell death (PCD) pathway and ultimately may lead to the death of the cells[105].

1.5.3 The role and nature of various antioxidants available to plants cells and involved in protection of cell from chilling induced oxidative stress.

Physiological and biochemical changes occur as part of cold acclimation that affect growth, water balance, accumulation of compatible solutes and antioxidant production in cells and thereby affect the ability of the plant to tolerate chilling temperatures. At times of chilling induced oxidative stress plants induce defense mechanisms including enzymatic superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxiredoxins (PRX) and non-enzymatic antioxidative compounds such as glutathione, flavonoids, carotenoids and tocopherols[109]. Most of the enzymatic and non-enzymatic antioxidants have been to found to induce in plants response to environmental stresses including drought, cold, salt, metal stress and water stress[109]. Enzymatic or non-enzymatic, antioxidants are involved in the reduction of ROS molecules by donating an electron and thereby stabilising the highly reactive molecule into a less harmful one and becoming oxidised themselves. The oxidised antioxidant is a lot more stable and less harmful to components of the cell. Hence, accumulation of antioxidants has been suggested to be associated with increased plants tolerance to abiotic stresses.

The enzymatic antioxidants (SOD, CAT and APX) are coded by small gene families where SOD's are involved in dismutation of superoxide anion into water and hydrogen peroxide. The hydrogen peroxide is then destroyed by catalase and peroxidase [110–112]. CAT's are a determining factor in the protection of photosynthetic cells from oxidative

stress and are therefore commonly found induced in chilling stress, drought and salt[113,114]. APX use ascorbate to dismutate hydrogen peroxide into water and, in the process, stimulate production of monodehydroascorbate reductase (MDAR). MDAR disproportionate into ascorbate and dehydroascorbate. Dehydroascorbate reductase uses glutathione (GSH) to reduce dehydroascorbate to ascorbate[109]. Transgenic rice plants and tobacco plants overexpressing OsAPX1a or tAPX gene, respectively showed enhanced tolerance to photooxidative damage during chilling stress[115,116]. Peroxiredoxins are dependent on thioredoxin for their function and work with ascorbate-glutathione cycle to dismutate hydrogen peroxide in plants exposed to different types of stresses[117].

Ascorbate-glutathione cycle and flavonoids are water soluble antioxidants while carotenoids and tocopherols are lipid soluble antioxidants[118–120]. The ascorbate and glutathione cycle is located in the stroma of the chloroplast are responsible in protecting photosynthetic cells from damage by oxidative stress[121,122]. Glutathione (GSH) is present in cellular components including cytosol, vacuole, chloroplast and endoplasmic reticulum[123]. GSH is involved in sulphur transport, induction of stress responsive genes and is essential for detoxification of xenobiotics in the chloroplast where it protects photosynthetic apparatus from oxidative damage[109,123].

Flavonoids and carotenoids are found in all plant foods and consist of ROS scavenging activity[124,125]. Accumulation of flavonoids resulted in increased tolerance to oxidative and drought stress conditons[126]. Carotenoids are divided into two classes that includes carotenes and xanthophylls where most of them consist of antioxidant activity[127]. Carotenoids increase visual attraction of plants and provide protection against photooxidative damage during photosynthesis[128]. Overexpression of carotenoids resulted in increased tolerance of *A. thaliana* to high light and high temperature stress[129] Finally, tocopherols are synthesized in all plants and are involved in protection

of chloroplast membrane by causing quenching of ROS and removal of lipid peroxides[130]. Accumulation of tocopherols has also been suggested to be associated with increased adaptation to low temperature stress[131]. Thus, accumulation of enzymatic and non-enzymatic antioxidants during chilling stress is thought to increase sensitive plants adaptability to chilling conditions.

Antioxidant activity is also found in ubiquitously produced polycationic nitrogenous compounds called polyamines such as spermidine, spermidine and putrescine[132]. Polyamines are involved in many physiological processes including cell growth, development and play a role in increasing tolerance to stress conditions[133]. Chilling tolerant plant varieties of bean, during chilling stress, induced the production of polyamines while chilling sensitive plant varieties did not [134]. These findings indicated the role of polyamines in plants chilling tolerance, and hence there has been growing interest in investigating the role of polyamines in enhancing plants tolerance towards chilling temperatures[135]. Pre-treatment of a chilling sensitive cucumber variety with spermidine during chilling stress reduced its hydrogen peroxide and NADPH oxidase content while untreated chilling sensitive cucumber did not show these changes[132]. In addition, spermidine foliar spray on rice increased antioxidant capacity significantly and reduced membrane damage by lipid peroxidation following cold[136]. Exogenous application of spermidine was also found to enter intact chloroplasts rapidly and protect the photosynthetic apparatus such as PSII on the thylakoid membranes from adverse effects of chilling stress in the spinach leaves[137]. These antioxidative effects of polyamines suggest its beneficial role in enhancing the plants tolerance towards chilling stress.

1.5.4 The role of COR genes in the protection of plant cells from damage by chilling temperature:

The protection from damage by chilling temperatures at a molecular level that includes the CBF genes inducing expression of target genes has been described above. The mechanism through which induced expression of the CBF regulon causes an increase in *A. thaliana* freezing tolerance is not yet fully understood but involves the expression of genes coding for polypeptides with cryoprotective activity [138,139]. For instance, COR15a codes for a chloroplast stromal protein called COR15am that causes changes in the intrinsic curvature of the inner membrane of the chloroplast envelope that defers membrane phase change from lamella phase to hexagonal II phase with increased permeability at freezing temperatures[139] (Figure 1.7). COR15am therefore stabilises the chloroplast envelope and reduces membrane damage at freezing temperatures in *A. thaliana*[139].

Tolerance to chilling temperatures is a quantitative trait and can be developed from the additive effect of expression of various cold-inducible genes[40]. Many of the cold-inducible genes encode proteins with known functions, but there are also other genes that code for, as yet, unidentified proteins[140]. According to Hannah *et al.* [141], the most overrepresented cold-inducible genes during short (12 h), medium (24 – 48 h) and long (> 48 h) term storage of *A. thaliana* at freezing temperatures were late embryogenesis abundant (LEA) genes. These are normally expressed in the late embryo development or during dehydration stress[142]. Dehydrins make up a unique group of LEA proteins called LEA II proteins and are also part of the CBF regulon such as COR15a, COR15b, XERO2, RD29A, RD29B, RAB18, COR47, ERD10, and ERD1[141,143]. Dehydrins are coded by the most expressed genes during drought stress or other environmental threats that can cause dehydration of cells such as cold, salt and heat stress [144]. Dehydrins contain high proportions of hydrophilic amino acids and also consist of a conserved amino acid sequence that is rich in lysine called the K segment [140]. The K-segment has the capability to form amphipathic α-helices upon a decrease in the dehydrin's hydration status [144]. The amphipathic α-helices allows dehydrins

to interact with partly dehydrated surfaces of proteins and biomembranes and prevent them from further dehydration[144]. When in aqueous solution, dehydrins are present in a random coil, forming maximum hydrogen bonds with the neighbouring water molecules and minimum hydrogen bonds with the neighbouring amino acids within the molecule[144]. The ability of the dehydrins to bind with water and accumulate during dehydration stress can assist in maintaining the cell volume and prevent it from collapse [145]. Some dehydrins also consist of large amounts of histidine and arginine amino acids that enable dehydrins to act as antioxidants. These amino acids interact with, and stabilise, ROS by becoming oxidised themselves and also bind to the metal ions (Co^{2+}, CU^{2+}, Fe^{2+}, Fe^{3+} Ni^{2+} and Zn^{2+}) to prevent them from causing further formation of ROS.

1.5.5 Injury to biological processes caused by chilling temperatures:

Several biological processes in chilling sensitive plants suffer from stress caused by chilling temperatures, among which are water relations, mineral nutrition, generation of reactive oxygen species (ROS), and carbohydrate metabolism.

1.5.5.1 Water relations:

At chilling temperatures, the water transportation from the roots to the shoots is reduced in response to reduced water absorption by the roots[87]. This disturbance in water transportation around the plant can lead to water deficit in the shoots and result in wilting of the leaves. In a study by Yu *et al.*[146] rice varieties cultivars, Somewake (chilling tolerant) and Wasetoitsu (chilling sensitive) were given a chilling treatment (7^0C) for 24 h and then allowed to recover for 24 h at ambient temperatures (28^0 C). In both the varieties, the relative water content, transpiration and root water uptake were reduced by chilling stress. However, when allowed to recover, Somewake was found to have made a full recovery in its leaf relative water content, transpiration and root water uptake by returning to initial values pre-chilling treatment but Wasetoitsu showed recovery only by 72.5%, 46%

and 65%, respectively. Aquaporins have been found to play important roles in water transport at times of exposure to chilling temperatures[146–148]. Aquaporins are water channels that play a role in facilitating water movement across the cell membrane[149]. *A. thaliana* protoplast membrane deficient in PIP1 aquaporins showed decrease in permeability to water by a factor of three (from 11 µm s^{-1} to 3 µm s^{-1})[150]. According to Kaldenhoff *et al*.[150] the increased root mass observed in PIP1 deficient *A. thaliana* may have occurred to compensate for the lack of water permeability and to ensure enough water supply to other parts of the plant. The finding of this study demonstrates the importance of aquaporins in water transportation around the plant. In addition, Yu *et al*. [146] studied the expression of 11 aquaporins in rice and found them to be downregulated at chilling temperatures but upregulated during the recovery phase at 28^{0}C for 24h. Yu *et al.* [146] concluded that aquaporins are water channels that help re-establish the water balance which is otherwise lost in chilling sensitive plants at chilling temperatures.

1.5.5.2 Mineral nutrition:

The nutrient status of the plant suffers during exposure to chilling temperatures due to reduced water uptake and its transportation from roots to shoots, leading to a general decrease in the nutrient content in the plants[87]. Chilling temperatures also results in reduced nitrate reductase activity that in turn reduces the incorporation of nitrogen into amino acids and proteins[24]. This also causes protein synthesis to be reduced and may contribute to reduced plant growth and development at chilling temperatures in *Azolla caroliniana* [151].

1.5.5.3 Carbohydrate Metabolism:

Carbohydrate metabolism involves synthesis, degradation and interconversion of other compounds into carbohydrates such as glucose and sucrose. Plants need carbohydrates to produce ATP to fuel the metabolic processes needed for their growth and development.

Processes of carbohydrate metabolism most affected by chilling temperatures include photosynthesis, respiration, and starch accumulation[152].

1.5.5.3.1 *Photosynthesis:*

Photosynthesis is reduced in warm-climate grown plants after storage in chilling temperatures[153]. Excessive light energy absorbed but not completely used by the photosynthetic apparatus causes generation of ROS. The newly formed ROS by light energy can damage photosystems (photooxidation) and thereby reduce photosynthetic capacity of the plant (photoinhibition). Hetherington *et al.*[154] reported that photoinhibition occurred in plants during their storage at chilling temperatures irrespective of their sensitivity towards chilling temperatures. However, the extent of photoinhibition in chilling resistant plants (barley, broad bean and pea) was measured to be about half of that observed in chilling sensitive (bean, cucumber, maize, and tomato) plants. Photoinhibition in plants was also observed to be dependent on temperature and light, increasing non-linearly with decreasing temperature and linearly with increasing light intensity[154]. Similarly, photo-oxidation is also dependent on the light intensity and oxygen availability. At low temperature, the metabolic rate is reduced, hence the majority of the light energy absorbed is not converted into chemical energy. The over-reduced electron transport chain leads to the generation of ROS[155]. The increased ROS then causes lipid peroxidation and compromises the integrity of thylakoid and other chloroplast membranes. Both PSI and PSII are affected by photooxidation, and photosynthetic pigments are also degraded causing the process of photosynthesis to be reduced.

Two classes of *Zea mays* genotypes with differing sensitivities to chilling temperature were grown at either 24^0C or 14^0C. At 14^0C, the process of photosynthesis was reduced in both the genotypes[156]. The chilling sensitive genotype, however, showed a lower content of chlorophyll a and b, reduced ratio of chlorophyll a to b, and an increased ratio of carotenoid to chlorophyll than the chilling tolerant genotype[156]. Therefore, it may be

possible that increased chlorophyll and carotenoid content are associated with chilling tolerance in plants too.

1.5.5.4 Respiration:

The response of the respiration process to chilling stress is very variable between species[87]. Respiration has been reported to decline at chilling temperatures due to the destruction of the structure of the mitochondria and inhibition of the enzymes involved in the process. However, the respiration rate has also been found to increase in its rate under chilling stress[157]. This increase in the respiration rate could be accounted for by the activation of the alternative respiratory pathway referred to as the alternative oxidative pathway (AOX). The increase in the respiration rate is believed to be an adaptive mechanism employed by the plant to prevent over-reduction of the electron transport chain. These alternative respiratory pathways, while producing less ATP than the original respiratory pathways, can decrease the damaging ROS produced in response to chilling stress [158].

1.5.5.5 Starch Metabolism

Starch is a polymer chain of several α-glucose units joined by glycosidic bonds. It is synthesised either as amylose or as amylopectin. Amylopectin is a branched polymer where chains are linked to each other through α-1, 6-glucosidic bonds on every 20-25 glucose units. The resulting molecule consists of a maximum of 100,000 glucosyl units. Amylose, on the other hand, is an unbranched chain and only consists of about 1000 glucosyl units. These polymers are stored as a carbohydrate reservoir in tissues including leaves, roots, seeds and tubers to provide fuel for metabolism and growth at times of carbohydrates deficiency. The starch content decreased in tomato chilling sensitive plants when kept in the dark and chilling conditions. The amount of starch content was parallel with tomato plants' chilling tolerance. When the starch content was lowest in the last hours of the dark phase then so was the tomato plants' chilling tolerance and *vice versa* [159]. On the other hand, the proportion of starch to sucrose that remains similar at 20^0C

was found to differ considerably at chilling temperatures. Starch content declined more rapidly and was barely detectable at 5-6^{0}C when compared to sucrose content. This was suggested to be due to lower inhibition on sucrose biosynthetic enzymes at chilling temperatures than on starch biosynthetic enzymes [160,161]. Sugars function as osmolytes, and their accumulation can prevent cellular dehydration by maintaining the osmotic difference between the cell and its environment [160].

Physiologically, at chilling temperatures, the catabolism in chilling sensitive plants appears to dominate over synthesis, using up all the resources available to promote and prolong its survival for as long as it can. However, if the stress persists and if the plant fails to build defence mechanisms to either adapt or defend itself against such harsh environmental conditions resource exhaustion will occur, eventually leading the plant to its death.

1.5.6 Molecular changes in chilling sensitive plants at chilling temperatures

The complete genome-sequenced, chilling-tolerant *A. thaliana* was used extensively to identify changes occurring in the acclimation processes to freezing temperatures [162]. Many essential molecular components such as the CBF pathway were identified as key regulators of acclimation to freezing temperatures in freezing sensitive plants. However, little is known about the changes occurring at a molecular and biochemical level in plants during acclimation to chilling temperatures[163]. Therefore, to identify molecular components involved in acclimation to chilling temperatures, chilling sensitive mutants of *A. thaliana* were created with increased sensitivity to chilling temperatures[163].

Chilling sensitive mutants of *A. thaliana* were sorted into different classes based on the changes they showed genetically and phenotypically after one week of treatment at chilling temperatures[164]. Class 1 chilling sensitive mutants showed a more severe phenotype than class 2 and class 3 chilling sensitive mutants. Class 1 mutants do not survive after only three days of treatment at chilling temperatures, and their rosette leaves

wilt, discolour and die. Class 2 and class 3 mutants are able to flower and set seed at chilling temperatures but class 2 mutants' rosette older leaves become chlorotic and wilt while the younger leaves remain unaffected. In class 3 mutants the rosette leaves develop chlorotic patches[164]. When comparing the genetic profile of 12 chilling sensitive mutants treated with or without chilling temperatures, 634 abnormally expressed genes were commonly found across all 12 chilling sensitive mutants[163]. The abnormally expressed genes were related to chloroplast function, lipid metabolism, carbohydrate metabolism and detoxification of ROS [163].

Hannah *et al.*[141] analysed the changes in the expression of genes involved in carbohydrate metabolism and lipid metabolism taking place in short (12 h or less), medium (24 to 48 h) and long term (more than 48 h) exposure of *A. thaliana* to chilling temperatures. After short-term exposure to chilling temperatures, the transporters of sucrose and phosphate located on the plasma, vacuolar and plastid membrane were upregulated, increasing the transportation of ions and sucrose, in, out and within the cell. This observation indicates the importance of sugar distribution around the cell in an initial response of *A. thaliana* to chilling temperature. The genes involved in the degradation of starch and sucrose were also found upregulated in short term storage of *A. thaliana* at chilling temperature. The products (glucose and fructose) of sucrose and starch degradation can increase the availability of substrates that can enter glycolysis and subsequently the TCA cycle, whose genes were later found upregulated in the medium to long term chilling response. According to Hannah *et al.*[141], the upregulation of glycolysis and TCA cycle may contribute to the increased metabolite content in *A. thaliana* when stored under chilling temperature[165,166], and function to protect the cells from damage by cold.

Hannah *et al*[141] showed that the genes involved in fatty acid synthesis, elongation and degradation were downregulated during medium to long term exposure to chilling temperatures. This was believed to be due to decreased demand in the biogenesis of cell membranes at times of reduced growth under chilling conditions[141]. Provart *et al.*[163] found

that in a chilling sensitive class 1 mutant there was reduced expression of FAD2, FAD3, FAD5, FAD6, FAD7, and FAD8. These desaturases carry out desaturation of different types of fatty acids (16:0, 16:3, 18:0, 18:2 and 18:1) that form part of the lipids present in the membranes of several organelles, including the chloroplast, thylakoid membrane, endoplasmic reticulum and mitochondria[167]. Fatty acids are synthesised largely in the chloroplast then transported to the endoplasmic reticulum and mitochondria. Defects in the FADS enzymes can prevent desaturation of fatty acids and increase the susceptibility of compartmental membranes to damage by chilling temperature. Mutants of FAD6 and FAD7 exposed to cold temperature resulted in leaf chlorosis, loss of photosynthesis, reduced growth and impaired chloroplast structure in *A. thaliana*[167]. This demonstrates the importance of desaturation of fatty acids in preventing the cell membranes phase transition from liquid to gel-like or solid state in delaying the subsequent damaging events from occurring.

Understanding the changes taking place in chilling sensitive plants at chilling temperatures at the physiological and molecular level can aid plant scientists in developing mechanisms that provide the chilling sensitive plants with greater tolerance, and thereby protection, from chilling temperatures.

1.6 Ways of improving chilling tolerance towards chilling temperatures:

There are a few approaches that have been used to improve chilling tolerance in chilling sensitive plants, and they include the application of abscisic acid, cold acclimation, reducing photoperiod and irradiation with far-red light or reducing the ratio of red to far red light.

1.6.1 Application of abscisic acid

Accumulation of abscisic acid (ABA) is a common response of plants to abiotic stress[168]. Application of ABA has been found to reduce chilling damage in crops in several studies

[169–171]. ABA induces chilling tolerance by relieving dehydration in plants at times of chilling stress[169]. ABA is known to cause this effect by inducing rapid stomatal closure to reduce water loss[170]. Pardossi *et al*[168] investigated whether the endogenously increased content of ABA plays a role in ameliorating water stress during chilling temperature, using bean seedlings. In the first few hours of the chilling treatment, the stomata of bean seedlings remained open, ABA content did not increase and the leaves wilted. However, after 24 h of chilling treatment, ABA content increased and the leaves rehydrated. After rehydration, the stomata remain closed, and the increased ABA content was higher in both roots and leaves of chilling-treated bean seedlings compared to seedlings kept at ambient temperature for six days. These findings suggest that endogenously accumulated content of ABA may play a role in relieving water stress and thereby reduce damage caused/induced by chilling temperature. Other than stomatal closure, many cold-regulated genes were induced by ABA including the transcription factors CBF1- CBF3, which in turn increases the expression of CBF target genes and consequently improve plant chilling tolerance [171].

1.6.2 Cold acclimation

Most cold-sensitive plants can develop tolerance towards chilling or freezing temperatures by being treated at moderately low, but non-freezing, temperatures in a process called cold acclimation[140]. Anderson *et al*.[172] demonstrated the effect of cold acclimation on the development of chilling tolerance in maize seedlings by growing them at three different chilling temperatures (4, 5 and 6^0C) and assessing their survival percentage after ten days. Growth and survival rates were much greater of maize seedlings across all three chilling temperatures when the seedlings were acclimated at 14^0C for three days before being grown at chilling temperatures. 79% of the seedlings survived after acclimation at 14^0C compared to 21% of the seedlings that survived treatment at 5^0C without prior acclimation at 14^0C. Koster and Lynch[173] found total soluble sugar content to have doubled after only two weeks of acclimation to cold temperatures in Puma Rye, and this was attributed mainly

to increase in the content of sucrose and raffinose content. Sucrose and raffinose are cryoprotectants and are able to provide protection to the cells against damage by chilling temperature [174].

Cold acclimation in plants is a complex process involving metabolic reprogramming and transcriptional changes [174,175]. During acclimation, temperate plants produce metabolites (soluble sugars, sugar alcohols and low molecular weight nitrogenous compounds) with cytoprotective activities, increase generation of COR proteins, dehydrin protein and heat shock proteins that together can stabilise biological membranes, proteins, and limit generation of ROS in the cell [174]. These effects protect the cell against damage from chilling temperatures, making the approach of cold acclimation a promising one in inducing chilling tolerance in chilling sensitive plants. However, introducing an application to cool pot herbs at suboptimal low temperature for few days is very expensive and not feasible to incorporate as part of the production chain in a commercial environment.

1.6.3 Photoperiod

Most plants sense the shortening of the days during autumn, accompanied by a gradual lowering of the temperature that acts as a cue for the onset of winter. These changes in the environment trigger the cold adapting mechanism in the plant to increase its tolerance towards the upcoming freezing temperature in winter conditions [176]. *A. thaliana* grown in short days increased its freezing tolerance by 2^0C when grown in short days for five weeks (8 h) compared to long days (16 h) for three weeks [177]. The effect of photoperiod on the plant's cold tolerance is mediated by phytochromes [178]. When the dark period of short day grown (8 h) *Cornus stolonifera* plants was interrupted with red light, the cold acclimation of the plant was inhibited. However, when red light treatment was soon followed by FR light, the cold acclimation was re-established suggesting that the cold tolerance in *Cornus stolonifera* was positively regulated by inactive phytochromes and negatively regulated by active phytochromes[178]. In addition, Lee and Thomashow[177] found short photoperiod (8 h) treated *A. thaliana* showed three to five fold higher expression of CBF genes than when

treated with long photoperiod (16 h), suggesting that CBF transcript expression is negatively regulated by the photoperiod.

Single mutant studies of *A. thaliana* of either bHLH transcription factor *pif4* or *pif7*, have indicated that they may act redundantly to repress the expression of CBF genes by binding on to the G-box in their promoter region and preventing their transcription from occurring [177]. Under long day conditions, the PIF4 and PIF7 levels accumulate but reduce when plants are grown in short day conditions, and this relieves suppression on the CBF pathway resulting in short day treated plants to develop greater freezing tolerance[67] (Figure 1.8 and 1.9). The expression and stability of PIF4 and PIF7 may be regulated by phyB. However, phyB is known to cause degradation of PIF4 but not of PIF7[179]. Therefore, it was proposed by Lee and Thomashow[177] that perhaps interaction of a PIF4 and PIF7 heterodimer with active phyB might prevent degradation of PIF4. However, this proposed mechanism does not fit with the finding of CBF repression in mutants of *pif7*. Hence, further work involving detailed analysis of PIF proteins present at the CBF locus under short day and long day grown seedlings is required to resolve the mechanism of phyB interaction with PIF4 and PIF7 when repressing the CBF genes expression. Photoperiod may be able to increase tolerance in freezing tolerant plants, and it could potentially be used to determine whether it can similarly increase chilling tolerance in chilling sensitive plants. However, shortening of photoperiod would require in built screens to reduce day length and lighting that makes this approach expensive and not viable. It is interesting to note that while at chilling temperatures the PIF4 and phytochromes levels are reduced and their effects on the development of the plant is depressed, the opposite occurs during warm season of the year. Recent findings have shown that Pfr and PIF4 are involved in sensing of warm temperatures in *A. thaliana* and are needed for proper hypocotyl elongation and induction of flowering at higher temperatures[180,181]. The opposite effect of phytochromes and PIF4 on the development of the plant during different seasons of the year, adds support to their involvement in plants responses to temperature stress. PIF4 and Pfr play an antagonistic

role during chilling tolerance stress but are important in mediating plants responses to warm temperature stress.

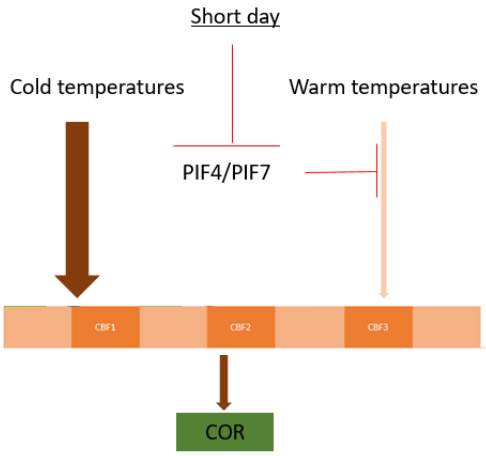


Figure 1.8 The photoperiodic regulation of CBF at warm temperatures in short days. Reproduced from Maibam *et al*[67]. PIF4 and PIF7 level is decreased under short day conditions and that relieves repression on CBF pathway and allows their transcription to occur. The CBF transcription factors subsequently induce expression of COR genes and increase plants' tolerance to cold temperatures.

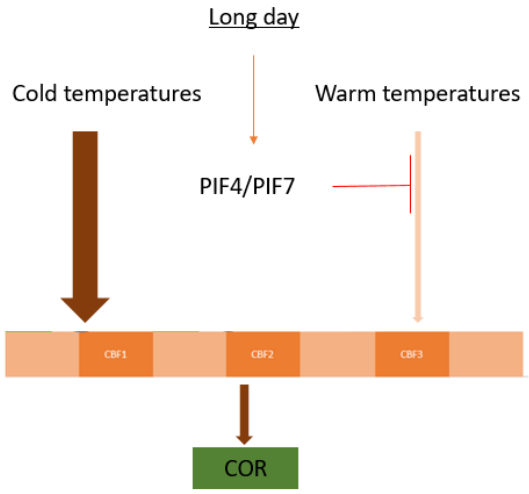


Figure 1.9 The regulation of CBF pathway in long day conditions at warm temperatures. PIF4 and PIF7 levels increase under long day conditions where PIF4 and PIF7 bind repress expression of CBF genes and prevent development of cold tolerance in plants.

1.6.4 Effect of light quality on plants responses towards chilling conditions

Light is required for the induction of various cold-responsive genes, and its quality is important in the regulation of the CBF pathway[182]. Light quality low in red to far red light (i.e. the ratio LR:FR) converts active phytochromes to inactive phytochromes and thereby relieves suppression on the CBF gene expression. The increased CBF gene expression, in turn, causes an increase in the expression of its target genes that has resulted in an increase tolerance in *A. thaliana* towards freezing temperatures [183]. The use of light quality to improve cold tolerance in plants is based on the phytochromes ability to influence the activation of the CBF pathway. However, the CBF pathway is also regulated by the circadian clock and the temperature of the environment. Hence, it is important to consider the effect of light, temperature and time of the day's effect on the regulation of CBF pathway, as such knowledge would be useful in identifying a suitable regime when the CBF pathway activation is at its maximum.

1.6.4.1 CBF pathway regulation by circadian clock, temperature of the environment and light quality

The CBF pathway is positively regulated by the circadian clock during the day by CCA1 and LHY but day light is rich in red light compared to FR light (High Red to Far Red ratio, HR:FR) and the phytochromes are in their active state (Pfr)[184]. The HR:FR light regime represses cold acclimation in plants by suppression of CBF genes expression caused by active phyB, PIF4 and PIF7 (Figure 1.10a) [177]. Hence, in daylight, the stimulation by CCA1 and LHY is balanced out by the repression from the light quality of the daylight. During the night the active phytochromes are gradually converted into inactive phytochromes and as a result, the repression on the CBF pathway from active phyB, PIF4 & PIF7 transcription factors is relieved[65,177]. There is, however, at the same time a loss of positive regulation of expression of CBF transcripts from the circadian clock in the night due to depletion of CCA1 and LHY, so there is still no CBF gene activation (Figure 1.10b)[65]. Hence, CBF genes expression is not

induced during the day or night when plants are grown in natural day/night cycles in warm conditions.

If the temperature of the environment falls under HR:FR light conditions during the day the CBF pathway is not only upregulated by the circadian clock but also by ICE1 and CAMTA3 that are transcription factors activated at times of low temperatures (Figure 1.10c)[77]. There is also suppression of the expression of CBF genes from active phyB, PIF4 and PIF7. However, the stimulation by CCA1, LHY, ICE1 and CAMTA3 on CBF gene expression[65] is likely to outweigh the suppression by phyB, PIF4 and PIF7 and cause the net effect to be upregulation of the CBF pathway (Figure 1.10c). In the night there is again a loss of the positive-acting CCA1 and LHY activity and the negative acting phyB, PIF4 and PIF7 but there is still upregulation of the CBF pathway caused by the activated transcription factors ICE1 and CAMTA3 (Figure 1.10d)[185]. Thus, the CBF genes under chilling conditions are strongly upregulated during both the day and the night in cold conditions[65].

In the vegetative shade, the light is relatively enriched in FR light resulting in the light having a low red to far red light ratio (LR:FR). The vegetative shade causes active phytochromes (Pfr) to be converted into inactive phytochromes (Pr) and thereby prevent the repression of CBF genes from occurring (Figure 1.10e) [19]. Even in warm days, the positive regulation of the CBF pathway by the circadian clock during the day and absence of repression by phyB, PIF4 and PIF7 tips the expression of CBF gene towards upregulation (Figure 1.10e). In the night, there is no stimulation from CCA1 and LHY on CBF gene regulation (Figure 1.10f) so in warm nights there is no upregulation once again. Thus, the vegetative shade that is low in red to far red light ratio can trigger CBF activation in warm conditions but only during the day. In the warm, and vegetative shade, the balance of CBF pathway regulation is tipped towards the lack of upregulation in the night but upregulation during the day (Figure 1.10f), corresponds to the pattern that was observed by Franklin and Whitelam in *A. thaliana*[127].

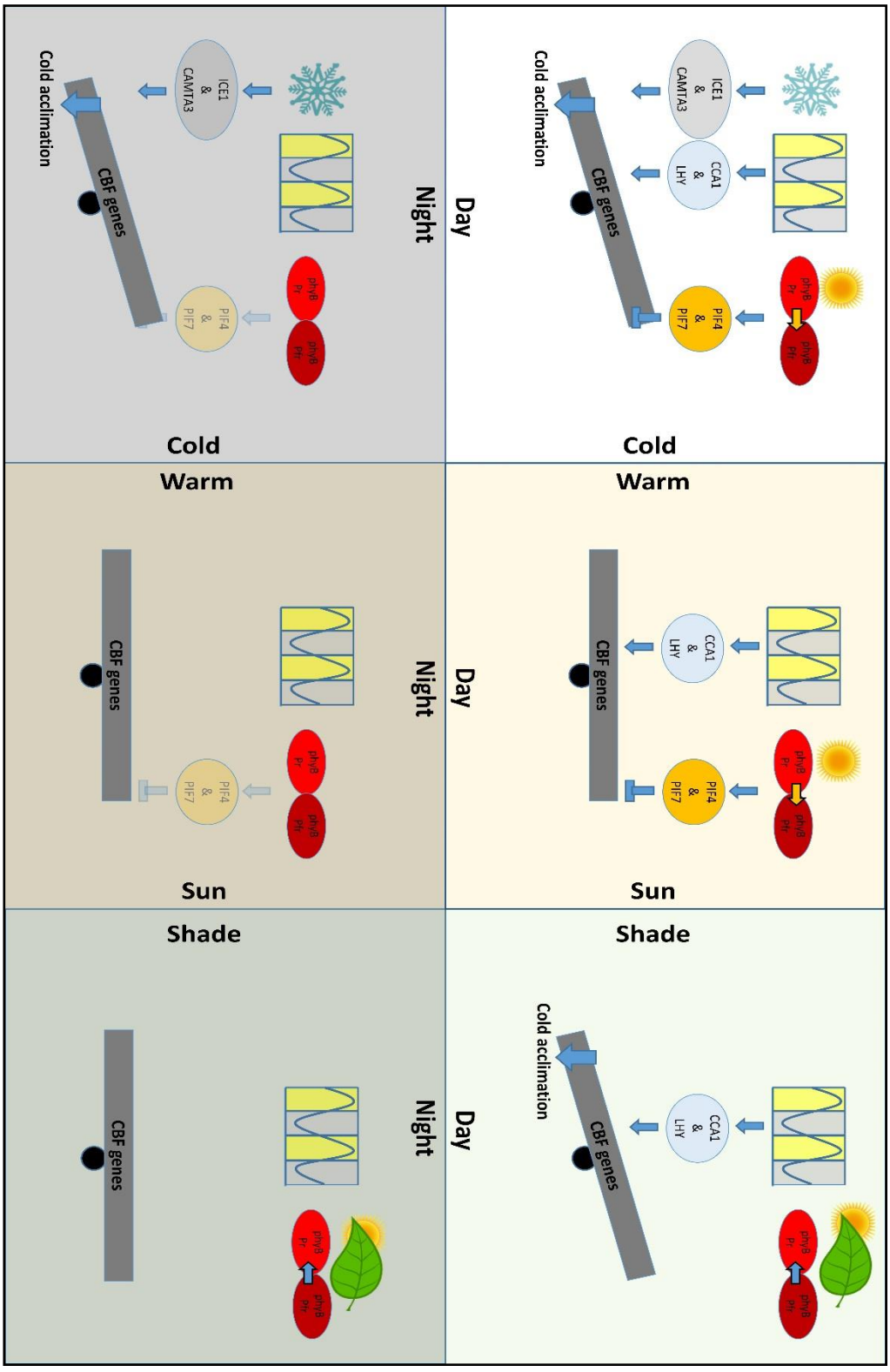


Figure 1.10 Proposed regulation of CBF pathway by warm temperature, light quality high red to far red ratio (HR:FR) and the circadian clock (a-b), low temperature, light quality (HR:FR) and the circadian clock (c-d), warm temperature, shade (LR:FR) light quality and circadian clock (e-f).

When considering the regulation of the CBF pathway by the circadian clock, the temperature of the environment and the light quality, it appears that LR:FR light treatment should be given during the day when the CBF pathway is also regulated positively by the circadian clock, as CCA1 and LHY promote the expression of the CBF genes and accumulate from ZT1-ZT3 [177]. Hence, if the LR:FR light treatment is given approximately around this time then it would convert the active phytochromes into inactive phytochromes. Pr-phyB would not interact with and stabilise the repressors, PIF4 and PIF7, and this would allow the expression of CBF genes to occur without any repression. Therefore, treatment of basil plants with LR:FR around the early to middle part each day is likely to provide an appropriate time window for optimal induction of CBF genes to occur at ambient temperatures.

In the study by Franklin and Whitelam [183] *A. thaliana* was treated with LR:FR light (ZT4-ZT8) on four consecutive days and then subjected to the experimental freezing conditions. Upon treatment with LR:FR light *A. thaliana* increased in its tolerance towards freezing temperatures when grown at temperatures (16^0C) higher than normally needed for cold acclimation. Greater survival rates and increased transcript abundance of *COR15a* and *COR15b* were found in *A. thaliana* plants treated with LR:FR light at 16^0C compared to plants treated with HR:FR light.

Use of LR:FR light to improve chilling tolerance in sensitive basil could be the most cost effective approach and, if effective, the light conditions could be easily replicated in the greenhouse settings of the herbs. Hence, if effective, LR:FR light treatment in the middle of the day would be given to pot grown basil for a few days just before its transportation stage to the distribution centre to induce chilling tolerance in chilling sensitive basil.

The overall aim of the project is to establish a way of modifying current basil growing conditions to improve its tolerance towards chilling conditions so that basil can be transported and displayed along with the other herbs at preferred chilling temperatures

To achieve the overall aim of the project the objectives include;

- To identify and quantify the changes occurring at the cellular level in basil in response to chilling temperatures, in order to establish the degree of its sensitivity towards chilling conditions (4^0C).
- To carry out RNA-sequencing analysis of basil treated with or without chilling temperatures in order to identify possible molecular pathways that could either potentially be targeted in the development of chilling tolerance or could explain the susceptibility of basil towards chilling temperatures.
- To investigate if LR:FR light treatment is effective in inducing chilling tolerance in sensitive basil by analysing the effect of the light treatment on the damage experienced by basil at a cellular level in response to chilling temperatures.
- If the treatment is successful, to carry out RNA-sequencing analysis of basil treated with or without LR:FR light at different time points during the treatment to investigate the molecular components, signalling pathways and the biological processes affected by the LR:FR light treatment and involved in inducing chilling tolerance in sensitive basil.

2 MATERIALS & METHODS

The plant materials used in all experiments were grown by Vitacress (West Sussex, UK). All experiments were carried out using a single basil variety, Marian (Enza Zaden, UK). The plants were grown until they were ready for the market which, in summer, was approximately 32 days and up to 52 days in winter.

2.1 Growth Substrate:

The growth substrate consisted of 100% grade 2 peat with additional TREF base fertilizer (TBF) fertiliser and was sourced from Estonia. Once the growth substrate was added with 36 seeds the seeds were then covered with more peat, the pots (9 cm) then received water from germination to pot spacing. After spacing, the pots were flooded and drained with full nutrition solution (pH 5.6). The electrical conductivity (EC) varying from 2.2 to 2.8 $\mu S.S^{-1}.m^{-1}$ depending on the frequency of watering. After germination, the pots were watered twice a day on sunny days to once every two days in cooler days. After spacing the pots were irrigated from twice a day to once in every three days depending on the prevailing temperature and sunlight.

The pots were grown in natural light throughout the year. However, when the natural light was less than 8000 lux (November to March) supplementary light from high-pressure sodium lamps (6000 lux) was added.

When determining the sensitivity of the basil plants towards chilling conditions, the plants were brought from Vitacress in the late afternoon and transferred to either the chilling temperatures (4^0C) or ambient temperatures (22^0C) under continuous darkness at ZT4 for 24 h. Plants were maintained under continuous dark conditions because these conditions reflect current transportation conditions. Basil plants responses to chilling temperatures were analysed 24h later by either physiological or molecular experiments.

2.2 Light treatment conditions

Basil pots from Vitacress were brought in the late afternoon and watered with tap water, by flooding, for 15 min before transferring them at ZT12 to darkness in Procema growth chambers. Plants received their respective light regime of either HR:FR light at a ratio of 5.11 and PAR 50 µmol m^{-2} s^{-1} or LR:FR light at a ratio of 0.16 and PAR of 47.29 µmol m^{-2} s^{-1}, at 22^0C. The ratio was measured in a 10 nm bandwidth around 660nm vs 10nm bandwidth around 730 nm. The control plants were irradiated with white light consisting of HR:FR ratio for 12 h from ZT0-ZT12 whereas the treatment plants were irradiated with white light consisting of HR:FR ratio for 12h from ZT0 – ZT12 but additional FR light was supplemented for 4 h from ZT4-ZT8. Hence, the plants in the treatment conditions received LR:FR light for 4 h in the middle of the day for each day of the four days of treatment. After four days of the treatment, the plants were transferred from HR:FR or LR:FR light to either continuous dark ambient conditions (22^0C) or continuous dark, chilling conditions (4^0C) at ZT4 for 24 h. The LR:FR light regime involved irradiating basil plants with additional FR lights from ZT4-ZT8 on top of white light.

After 24 h of chilling temperature or ambient temperature treatment, plants were harvested and their tissues (leaves) frozen in liquid N_2. Tissues were then stored at -80^0C for their subsequent physiological and molecular experiments; fresh tissues were also used immediately for some physiological experiments such as electrolyte leakage assay and DAB assay.

2.3 Environmental conditions that basil plants were exposed to for their subsequent high-throughput Illumina RNA sequencing.

Three separate RNA sequencing experiments of basil tissues were carried out. The first one was carried out to determine the transcriptomic changes in basil tissue in response to chilling temperatures and the second RNA sequencing experiment was carried out to

determine transcriptomic changes in response to irradiation with LR:FR light vs HR:FR light treatment at two different time points (ZT6 and ZT10). Lastly, the third RNA sequencing of basil material was carried out to elucidate transcriptomic changes occurring in basil tissue in response to chilling temperatures when pre-treated with HR:FR light or LR:FR light at ambient conditions for four days.

2.3.1.1 Environmental conditions of basil plants that were used for RNA sequencing to determine the transcriptomic changes in response to chilling temperature:

Plant material grown in green houses of Vitacress during the summer period were obtained and subjected to the experimental conditions. These conditions comprised 12 pots of herbs in a 12h photoperiod at a light intensity of 25 mol m^{-2} s^{-1} being exposed either to 24h of ambient conditions (22^0C) or 24h of cold conditions (4^0C). The plants were transferred to cold at ZT23 and harvested at ZT23. Top most expanded leaves were harvested in liquid nitrogen and stored at -80^0C for subsequent RNA extraction.

2.3.1.2 Environmental conditions that basil plants were exposed to for use in RNA sequencing to determine transcriptomic changes occurring in response to LR:FR light irradiation from ZT4-ZT8.

Plants were exposed to either HR:FR light (5.11) or LR:FR light (0.16) conditions for four days at ambient temperature (22^0C) with a 12h photoperiod. Plants irradiated with LR:FR light were irradiated with white light on top of FR light from ZT4-ZT8 on each of the four days. The plant material was harvested on the fourth day of the treatment at two different time points (ZT6 and ZT10). The plant material was immediately frozen in liquid N_2 then stored at -80^0C for subsequent RNA extraction.

2.3.1.3 Environmental conditions of basil plants that were used for RNA sequencing to determine basil's transcriptomic response to chilling temperatures when pre-treated with LR:FR light.

On the fifth day, the basil plants from their different light conditions (HR:FR or LR:FR) at ambient temperatures were transferred at ZT4 to either ambient temperature or chilling temperature under continuous darkness for 24 h. The basil plants were harvested from chilling and ambient conditions 24 h later (on the sixth day) at ZT4. The plant material was harvested in liquid N_2 then stored at -80^{0}C for subsequent RNA extraction.

2.4 Physiological experiments:

Basil plants were analysed for the effect of exposure to chilling temperatures by carrying out assays on the treated basil tissues as follows:

2.4.1 Electrolyte leakage assay:

The electrolyte leakage assay was modified from the method described by Campos *et al.*[186] and Bajji *et al.*[187]. Campos *et al.*[186] and Bajji *et al.*[187], both express the electrolyte leakage values measured as a percentage of total electrolytes where leaf discs were cut out from the entire leaves and immersed in distilled water. The electrolyte conductivity values of the water was measured immediately as initial electrolyte leakage reading (ELi). The electrolyte conductivity values were measured again after specific periods of time that ranged from every 5 minutes in the study by Bajji *et al.*[187] to 22h in the study by Campos *et al.*[186]. This reading was referred to as final electrolyte leakage (ELf). The samples were then autoclaved, and another reading was taken to give total electrolyte leakage values (ELt). These readings were then used to express electrolyte leakage from tissues as a percentage using the formula:

Relative electrolyte leakage (%) $= \frac{EL_f - EL_i}{EL_t - EL_i} X\ 100$

The two protocols of Campos *et al.*[186] and Bajji *et al.*[187] were adopted for this study, but the protocol was first validated before being used for determining chilling injury on basil leaves exposed to chilling temperature of 4^0C.

2.4.1.1 Electrolyte leakage assay – Version 1

Basil leaf discs were originally used for measuring electrical conductivity by cutting them out using a cork borer. However, results from protocols involving the use of leaf discs produced results with large variability between samples (data not shown) and therefore use of leaf discs for the electrolyte leakage assay was replaced by the use of entire leaves. To determine if the electrolyte leakage assay was effective in determining chilling injury on the damaged tissues, basil was exposed to an extreme duration of chilling temperature i.e. from 0 h to 48 h. The individual leaves removed were photographed and subjected to the assay. The assay involved measuring the conductivity of a single leaf in distilled water of 20 ml in a 50 ml Falcon tube using an Accument water proof conductivity meter (Fisher Scientific, UK). The ELi was measured immediately after the leaf was immersed in water. This was followed by ELf measurement after 12 h incubation on a shaker Infors AG, (Bottmingen, Switzerland) at 40 RPM. The ELt was measured after samples were rapidly frozen using liquid N_2 and then thawed.

2.4.1.2 Electrolyte leakage assay – version 2

The six top most fully expanded leaves from the basil pot were removed and immersed in 100 ml of distilled water in crystallizing dishes (150 ml). The leaves from ambient treated basil were removed and immersed in water at ambient temperatures, and leaves from basil exposed to chilling temperatures had their leaves removed and immersed in water before the samples were taken out from their respective chilling conditions out into the ambient conditions. The leaves in crystalline dishes were separated by a mesh into two layers of three leaves and an additional second mesh was placed on top of the second layer to prevent the leaves from rising above the water. The crystalline dishes with leaves were

then incubated at ambient temperatures in the dark and on the shaker Infors AG (Bottmingen, Switzerland) at the speed of 40 RPM. The electrolyte conductivity was measured immediately as ELi. After 12h of incubation in distilled water, the final electrolyte leakage reading was taken as ELf. The samples were then autoclaved at 120^{0}C for 30min to obtain the total electrolyte leakage values as ELt. The readings taken at different times were then used to express the electrolyte leakage from basil leaves as a percentage.

2.4.2 Lipid peroxidation protocol - 2-thiobarbituric acid (TBA) assay

The lipid peroxidation assay was carried out according to Hodges *et al.*[188] with slight modifications. Tissues for lipid peroxidation were weighed at harvest (fresh weight) and placed in 15 ml tubes then transferred into liquid nitrogen for snap freezing. The tissues were then transferred to -80^{0}C for subsequent storage. For extraction of Malondealdehyde (MDA) from the plant material, the tissues were transferred from -80^{0}C into a Dewar of liquid nitrogen. The tissues were ground in 5 ml of 5% Trichloroacetic acid (TCA) Sigma-Aldrich Company Ltd. (Dorset, UK) then centrifuged at 5000 x g (Eppendorf centrifuge, 5810R) for 15 min at 4^{0}C. The supernatant, containing the MDA, was stored at -20^{0}C for subsequent analysis.

From 2 ml of the supernatant, 1 ml was added to 1 ml of 5% of 2-Thiobarbituric acid (TBA*)* Sigma-Aldrich Company Ltd. (Dorset, UK) and the remaining 1 ml of supernatant kept without the TBA. The samples were vigorously mixed and heated at 95^{0}C for 30 min. The samples were transferred and kept on ice for at least 10 min to stop the reaction. Once cooled, the sample tubes were centrifuged again at 5000 x g for 10 min. 300 ul of these samples was then loaded into a 96 well plate where the absorbance was read at 532 nm. For greater accuracy of MDA detection further the absorbance values at two additional wavelengths (440 nm and 660 nm) was recorded[188]. This is due to 440 nm being the wavelength that is maximally absorbed by carbohydrates whereas 660 nm is the wavelength that is maximally absorbed by the anthocyanins. The absorbance of the

samples was therefore measured at these three different wavelengths with and without the addition of TBA, and the MDA equivalents were calculated according to the method described by Hodges *et al.*[188] from the absorbance readings in the following manner:

1) $[(Abs\ 532_{+TBA} - Abs\ 600_{+TBA}) - (Abs\ 532_{-TBA} - Abs600_{-TBA})] = A$

2) $[(Abs\ 440_{+TBA} - Abs\ 600_{+TBA})\ 0.0571] = B$

3) MDA equivalents (nmol. $ml^{-}1$) = $(A - B/157\ 000)10^6$

2.4.3 Antioxidants assay

The total water soluble antioxidant content was measured using leaves of basil. The antioxidants were extracted from the leaves followed by their assay for antioxidants activity using the Ferric reducing ability of plasma (FRAP) assay described by Benzie and Strain[189].

2.4.3.1 Extraction of material:

The top most fully expanded leaves were weighed then immediately frozen in liquid nitrogen in a 1.5 ml Eppendorf tube. The samples were stored at -80^{0}C, until assayed. The samples from the -80^{0}C were removed and ground to a fine powder in a pestle and mortar with sand, 0.6 ml of acetate buffer (300 mM, pH 7.6) was added to the mortar to transfer the lysate to a new 1.5 ml Eppendorf tube. A further 0.6 ml was added to the mortar to recover the remaining lysate from the mortar and added to the same Eppendorf tube. The lysate was then centrifuged at 31175.43 x g for 4 min, and the supernatant containing the water soluble antioxidants was transferred into a clean 1.5 ml Eppendorf tube. These samples, containing only the supernatants were then stored at -20^{0}C for subsequent analysis to be carried out using the FRAP assay.

2.4.3.2 Assay of water soluble antioxidants from leaves

The samples were assayed for quantification of antioxidant activity using the FRAP assay described by Benzie and Strain[189]. The antioxidant activity was measured using a 96 well microtiter plate. Antioxidant contents were compared to ascorbic acid using freshly prepared FRAP solution (25 ml of acetate buffer, 2.5 ml of 20 mM Ferric chloride - Iron (II) chloride hexadihydrate AnalaR Normapur (Haarlem, Netherlands) and 2.5 ml of 10 mM ,4,6-Tripyridyl-s-Triazine (TPTZ) in 40 mM HCl Sigma-Aldrich Company Ltd. (Dorset, UK)). To 30 ul of the sample or standard 300 µl of FRAP solution was added. The Fe^{3+} from the FRAP reagent is reduced to Fe^{2+} from the sample or the standard solution that resulted in the production of the blue colour. The intensity of this blue colour was measured in a microtiter plate spectrophotometer SpectraMax Plus 384 at 590 nm at ambient temperatures. Standard curves with correlation coefficients of not less than 0.999 were used to quantify the amount of antioxidants from the experimental samples. Once assayed, the antioxidant contents were normalized by fresh weight.

2.4.4 Chlorophyll fluorescence:

Basil seeds were sterilised by incubating them in 50% household bleach (Domestos*)* for 60min and then rinsed thoroughly with sterilised water. The sterilised seeds were then grown on solid agar media.

2.4.4.1 Culture media:

The growth medium consisted of 0.8% (w/v) high gel strength agar Sigma-Aldrich Company Ltd. (Dorset, UK) and 4.41 g of Murashige and Skoog nutrient medium including Gamborg B5 vitamins Duchefa Biochemie (Haarlem, Netherlands) in 1 L of sterilised water. The pH was adjusted to 5.6-5.7 with 0.1 M KOH, and the medium was autoclaved at 120^0C for 20 min. Once the agar cooled to being just hand hot, the medium was poured into Corning

plastic tissue culture dishes (100 mm x 20 mm) Sigma-Aldrich Company Ltd (Dorset, UK). Once the agar had solidified, 30 sterilised basil seeds were placed on the agar surface.

The petri dishes were closed with lids and sealed with Parafilm 3M, Sigma-Aldrich Company Ltd company (Dorset UK) to prevent any evaporation. The petri dishes with seeds were covered in aluminium foil for 72 h to provide complete darkness for the seeds to germinate at 22^0C. Following germination, the aluminium foil was removed from the petri dishes and the seeds were allowed to grow in continuous white light at 100 µmol m^{-2} s^{-1} for seven days. The seedlings were then exposed to 12 h photoperiod for three days. On the fourth day, the seedlings were transferred to either continuous dark and ambient temperature or continuous dark and cold conditions (4^0C) for 24 h.

2.4.4.2 Chlorophyll fluorescence measurement using NightOWL

Chlorophyll fluorescence from these plates was measured using NightOWL LB 983 *in vivo* Imaging System (Berthold Technologies UK, Harpenden, UK). The NightOwl imager measures the light emitted by the organisms non-invasively. After placing the plates in the chamber, the seedlings were irradiated with high-intensity blue light for 5 min. The light was switched off, and the pictures were taken immediately in darkness for 1 min to capture the fluorescence given off by the seedlings. Fluorescence values were obtained using Winlight 2.17 software (Berthold Technologies UK, Harpenden, UK).

The fluorescence yield was divided by the surface area to give delayed chlorophyll fluorescence yield from seedlings treated with or without the cold.

2.4.5 Reactive oxygen species assay

In this assay, the presence of hydrogen peroxide was detected via staining of basil tissues with 3, 3'-diaminobenzidine (DAB). The assay was carried out according to the protocol described by Daudi and Brien[190]. In this assay, a brown precipitate formed when DAB was oxidized by H_2O_3 in the presence of heam containing proteins (peroxidase).

2.4.5.1 Preparation of DAB staining solution

1mg/ml or 0.1% of DAB was prepared with sterile H_2O and the pH was reduced to 3.0 with 0.2M HCl and stored in the dark. 0.05% (v/v) of Tween 20 and 200mM Na_2HPO_4 (sodium phosphate), (Sigma-Aldrich Company Ltd., Dorset, UK) was added to the DAB solution to generate a final concentration of 10mM Na_2HPO_4.

2.4.5.2 Staining leaves with DAB solution

Basil leaves exposed to either 24 h of continuous darkness and ambient temperature or continuous darkness and cold temperature were removed and placed in a petri dish. Three leaves per petri dish were then immersed in 25 ml DAB solution. Leaves inside the petri dish were vacuum infiltrated for 5 min then the dishes were covered with aluminium foil and incubated at 30^0C in a Sanyo incubator for 4-5h on a Skyline shaker DOS-20M (ELMI, California, USA) at 53 RPM.

2.4.5.3 Bleaching of leaves

Following incubation, the leaves were drained of DAB solution and washed by immersion of leaves with bleaching solution (ethanol: acetic acid: glycerol = 3:1:1) for 15-20min in a water bath at 95^0C (Grant, SUB14). The leaves were drained from the bleaching solution, and the step was repeated but for 30min at 95^0C. Following this, the leaves were then rinsed with water. All leaves were photographed under uniform light and height conditions using Nikon D5200 camera fitted with an 18-55 mm lens. For calculation of ROS accumulation in (%), the ImageJ software was used to measure the area of the entire leaf and the colour threshold tool to measure the area of the leaf covered by the brown precipitate in pixels.

Detection of ROS in basil leaf was then expressed as a percentage using the following formula:

$$\text{Detection of ROS per leaf } (H_2O_3)\ (\%) = \frac{\text{Leaf area with brown precipitate}}{\text{Entire leaf area}} \text{ X } 100$$

2.4.6 Relative water content:

Relative water content (RWC) of leaves was measured in the way described by Yu *et al.*[146].The leaves of basil plants irradiated with either HR:FR light or LR:FR light were weighed immediately after exposure to 24 h of continuous darkness at ambient conditions or continuous darkness at chilling conditions (4^0C). The freshly weighed leaves were then placed in between two filter papers drenched in (25 ml) of deionised water in a petri dish that was sealed and subsequently transferred to chilling temperature (4^0C) for 12 h. The turgid weight (TW) of the leaves after 12 h was measured and then leaves were then transferred to 80^0C for 24 h for measurement of their dry weight (DW).

The relative water content of the leaves was then calculated in the following manner and expressed as a percentage:

$$\text{RWC (\%)} = \frac{FW - DW}{TW - DW} \times 100$$

2.4.7 Transpiration:

The transpiration rate of basil plants that were treated with HR:FR light or LR:FR light regime were measured for the first 180 min of their exposure to either continuous darkness ambient or continuous darkness chilling conditions. The transpiration of the plants was measured using a potometer (PXF150) *Jaytec* (East Sussex, UK). The rate of the transpiration was measured by recording the movement of the air bubble in the potometer in distance of mm by time in minutes. The rate of transpiration of plants was measured while the plants were present at the chilling temperatures or at the ambient temperatures. Measuring the transpiration rate after removal from 4^0C could have caused the plant to recover and transpire as it would have at ambient temperature. Therefore, in order to prevent the transpiration results from being affected by the ambient temperature, the impact of chilling temperature Vs ambient temperature was assessed by measuring the

transpiration rate of basil plants while they were present in either cold or ambient conditions.

$$\text{Rate of transpiration} = \frac{\text{Distance travelled by air bubble in mm}}{\text{time in min}}$$

2.5 Molecular experiments:

2.5.1 Extraction of total RNA from basil tissues for RNA sequencing:

Total RNA was extracted from leaf tissues of basil using an RNAeasy plant mini kit (Qiagen, UK). Total RNA was extracted from at least five biological replicates for each environmental condition and was pooled into one sample for each condition. The RNeasy silica-membrane technology used in the protocol efficiently removes most of the DNA without DNase treatment. Hence, the extracted RNA samples were not treated with DNase. Each pooled RNA samples integrity and purity were checked by carrying out RNA gel electrophoresis on the extracted RNA samples (Figure 2.1 and 2.2), and by measuring the absorbance of RNA samples at 260 nm, 280 nm and 230 nm (Table 2.1) using a Nanodrop spectrophotometer ND-1000 (Qiagen, UK). 1 μg of RNA was used to synthesize cDNA, and subsequently used in a qPCR reaction (see synthesis of cDNA, primer design and qPCR sections below).

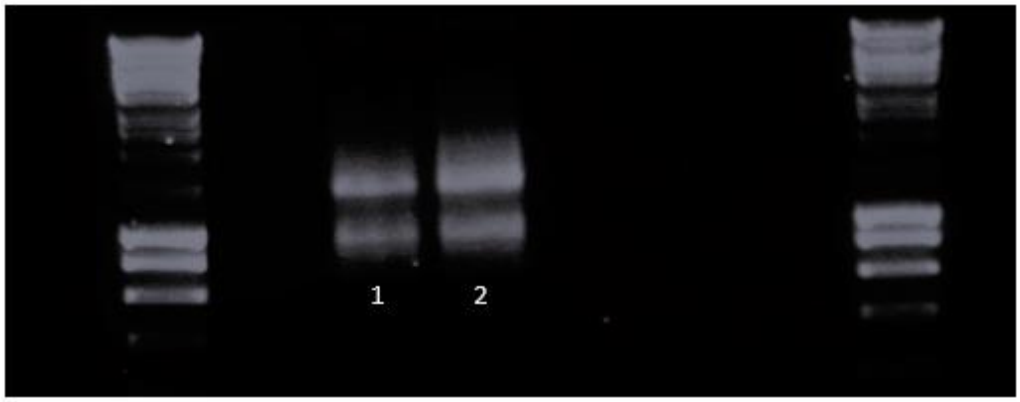


Figure 2.1 RNA gel electrophoresis of extracted RNA samples of basil and used for the first RNA sequencing experiment. The two samples (1-2) from left to right are (1) treated at ambient temperature for 24 h under 12 h photoperiod with HR:FR light or (2) treated at chilling temperature for 24 h under 12 h photoperiod with HR:FR light.

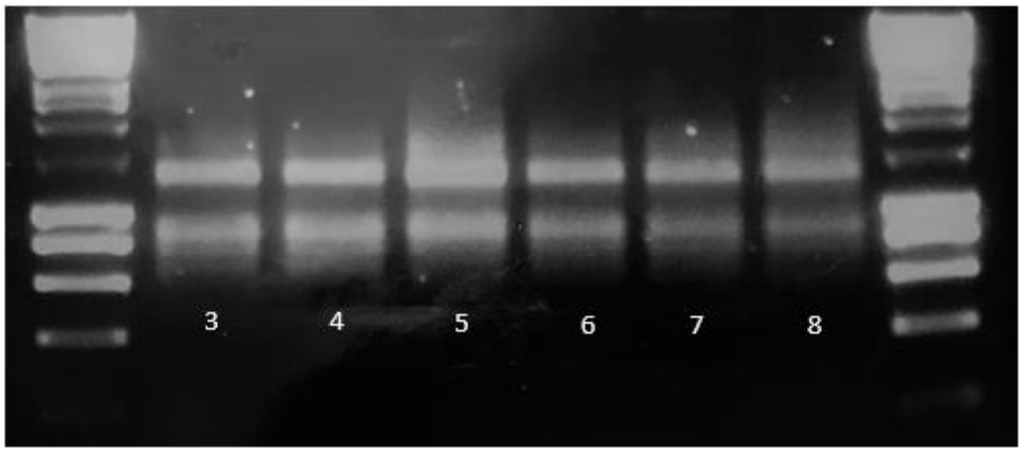


Figure 2.2 RNA gel electrophoresis of six samples (total RNA) (3-8) used for the second and third RNA sequencing experiment. From left to right, samples including 3, 4, 5 and 6 were all treated under 12 photoperiod. 3-6 samples were all harvested on the fourth day of the light treatment but at different time points and from different light conditions. From left to right are total RNA samples extracted from basil leaves treated either (3) at ambient temperatures for 24 h, under HR:FR light and harvested at ZT6 or (4) 24 h ambient temperature treated under LR:FR light and harvested at ZT6 (5) 24 h of ambient temperature treated basil plants under HR:FR light harvested at ZT10 or (6) 24 h of ambient temperature treated under LR:FR light harvested at ZT10, respectively. Finally, the last two samples (7-8) used in the third RNA sequencing experiment, include (7) 24 h

of chilling temperature treated basil under continuous darkness but pretreated with either HR:FR light or (8) LR:FR light under 12 h photoperiod for four days.

All samples from 1-8 used for the three different RNA sequencing experiment yielded two sharp bands (28S and 16S) in a 2:1 ratio indicating that the extracted total RNA from the samples were intact[191].

RNA sequencing experiment	Samples	Temperature for 24 h	Light treatment	Photoperiod	Sample harvest hr	ng/µl	260:280	260:230
First	1	22^{0}C	HR:FR	12 h	ZT23	457.81	1.98	2.03
	2	4^{0}C	HR:FR	12 h	ZT23	324.57	2.08	1.73
Second	3	22^{0}C	HR:FR for four days	12 h	ZT6 of fourth day	304.9	2.11	1.88
	4	22^{0}C	LR:FR for four days	12 h	ZT6 of fourth day	323.9	2.13	1.9
	5	22^{0}C	HR:FR for foour days	12 h	ZT10 of fourth day	265.1	2.14	1.96
	6	22^{0}C	LR:FR for four days	12 h	ZT10 of fourth day	214.5	2.12	2.11
Third	7	4^{0}C	HR:FR (Pre-treated)*	24 h darkness	ZT24 of fifth day	223.8	2.15	2.01
	8	4^{0}C	LR:FR (Pre-treated)*	24 h darkness	ZT24 of fifth day	321.7	2.11	2.01

Table 2.1 The ratio of absorbance at 260 nm and 280 nm and 260 nm and 230 nm and the estimated quantity of RNA (ng/ul) of samples 1 - 8 used for the three separate RNA sequencing experiment.

Nucleic acids including DNA and RNA absorb UV light at 260 nm and proteins at 280 nm[192]. The ratio of 260:280 nm was used to obtain an indication of the RNA purity from proteins. A ratio of ≥ 2.0 represents the high purity of the RNA sample where as a ratio of ≥ 1.8 signifies purity of the DNA sample[193]. The values provided in Table 2.1 represents A260:280 ratios of the RNA samples that are all ≥ 2 indicating that the extracted total RNA samples were of high purity.

Other compounds such as salts, carbohydrates, peptides and phenol present in the lysis buffers of most RNA extraction protocols absorb UV light strongly at 230nm[194]. Hence, the A260:230 ratios was also measured as the second parameters to indicate the purity of RNA from salts, phenols and other compounds. According to Ahlfen and Schlumpberger[194], an A260:230 ratio of ≥ 2.0 is indicative of highly pure RNA sample. In Table 2.1, all of the

samples consisted of A260:230 ratio ≥ 2.0, excluding sample 2 and 3, yield a ratio of 1.73 and 1.88. According to Ahlfen and Schlumpberger[194], high absorbance at 230 nm is almost always due to the presence of guanidine thiocyanate salt that absorbs very strongly at 230 nm. Experiments carried out by Ahlfen and Schlumpberger[194] have shown that the concentrations of guanidine thiocyanate salt as high as 100 mM do not affect qPCR experiment or similar sequencing experiments. Sample 2 yielded an absorbance ratio of 1.73 that corresponds to guanidine salt concentration of less than 0.1 mM. However, a more important factor to consider is the amount of contaminant that is passed onto the downstream reaction rather than the absorbance ratio. Hence, cDNA was synthesized and qPCR carried out using primers for the methionine synthase gene for samples 1, and 2 and UBQ7 primers were used for samples 3 – 8. Methionine synthase gene was one of the few basil genes sequenced at the time before the current RNA sequencing experiments were carried out. Hence, methionine synthase gene sequence was obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/nuccore/JN587800.1) nucleotide database and used to design primers. After the first RNA sequencing experiment, the UBQ7 gene was amplified by qPCR from the remaining six samples (3-8) that were used for the second and third RNA sequencing experiments.

The Ct values obtained from the amplification of methionine synthase or TUB6 did not vary by even one cycle in duplicate replicates of each sample and a single melt curve was obtained in all samples 1-8. The consistent Ct values and single melt curves with only one peak represents that the cDNA synthesized from RNA samples sent for sequencing to be of good quality and free from gDNA contaminants.

2.5.2 mRNA sequencing of basil exposed to different environmental conditions:

All samples were sequenced in the Core Cleveland Clinic facility, Cleveland Ohio by Dr Sandra Smieszek. The total RNA was purified to obtain mRNA using the protocol described by Wang *et al*.[195]. The mRNA was then subjected to paired end high throughput Illumina Hiseq2500 sequencing. The RNA sequencing analysis such as obtaining raw reads, read alignment and quantification were carried out by Dr Sandra Smieszek. The raw reads of basil were mapped to *A. thaliana's* transcriptome for the first RNA sequencing experiment. A *de novo* assembly was constructed using Trinity software (http://trinityrnaseq.github.io) for the second and third RNA sequencing experiment. The Trinity software was used for construction of basil's RNA *de novo* assembly as it represents a novel method for the efficient and robust reconstruction of transcriptomes from RNA-seq data. The error rate was assessed by testing with BLAST alignment of individual reads. The majority of analysis was carried out with iPLANT (aka Cyverse) and with Galaxy tools by Dr Sandra Smeiszek. Once the reads were reconstructed, the next steps involving calculation of differential gene expression, annotation of the genes, gene ontology analysis of differentially expressed genes from the RNA sequenced data, KEGG pathway analysis and visualisation of the differentially expressed genes effect on the metabolic pathways of the primary metabolism - were carried out by the author of this thesis.

2.5.3 Transcriptome assembly and calculation of differential gene expression.

The Fragments Per Kilobase of transcript per Million (FPKM) values for each transcript or contig was used to calculate differential gene expression in the following manner.

Differential gene expression (DGE) logged to the base of 2 = Log2 (treated FPKM) – log2 (control FPKM). The difference is the differential gene expression of values of transcripts of treated compared to control in logged form. The differential expressed gene value in log

form can be expressed in normal numbers by unlogging the DGE logged value. This can be done by carrying out the calculation of 2 to the power of logged value (2^DGE value).

2.5.4 Annotation of basil's unigenes and analysis of assembled basil transcripts data:

The identities of 8000 transcripts generated from the Trinity software were established using the programme nucleotide basic local alignment search tool (BLASTn)[196] (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The entire batch of 8000 sequences was uploaded on to BLASTn to search for subject queries that shared significant homology with that of the query sequence in reference RNA sequences (refseq_rna) database against *A. thaliana* organism (taxid: 3702). The BLASTn output for each query sequence included up to 100 subject sequences that shared similarity with that of the query sequence. The other details associated with the subject sequence that also formed part of the BLASTn output include, the subject sequence GenBank accession number, identity (%) that it shares with the query sequence, E-value and bit score.

E value and bit score are statistical estimates used by BLASTn to establish whether the similarity found between the sequences is by chance or not. E value by NCBI BLASTn software is calculated using the formula $E=mn2^{-S}$, where m refers to the length of the query sequence, n is the size of the data base and S signifies the raw score of the alignment[197]. A calculated e-value of 0 represents that the similarity found between the two sequences is significant while a value of 1 suggests for the similarity found to have occurred purely by chance[198]. Bit score is used in addition to e value where it is a normalised value of raw pairwise alignment score and not dependent on the size of the data base or the length of the query sequence[199]. Bit score is calculated using the following formula where λ, K and R are the statistical parameters:

$$S = \frac{\lambda R - \ln K}{\ln 2},$$

2.5.4.1 *Rationale of statistical estimates used to infer significance between two similar nucleotide sequences:*

Professor William Pearson's group at the University Of Virginia School Of Medicine have worked on developing methods for identifying distantly related protein sequences since the development of the FASTP program in 1985. According to Pearson 2014, a bit score of 40 for a protein alignment is significant in a database with 7000 entries[200]. Each increase of 10 bit score in a database increases the statistical significance by 1000 fold. Hence, a bit score of 50 would represent statistical significance in a database with entries of less than 7 million entries[200]. The refseq_RNA nucleotide database used in this study is 77 million sequences large[201]. Hence, a bit score of 80 was used to determine the similarities between two nucleotide sequences as significant.

After obtaining the BLASTn output of 8000 nucleotide sequences alignment, a Microsoft Excel macro was constructed to pick out the top hit for each sequence (Appendix 2.1). These sequences were filtered and removed if they possessed a bit score of less than 80. The ref seq accession numbers were used to obtain unigene ID's such as its Arabidopsis Genome Initiative (AGI) ID, description and symbol information from the software gprofiler: gene ID converter[202] (http://biit.cs.ut.ee/gprofiler/gconvert.cgi). Once the basil's transcripts were blasted against *A. thaliana*, annotated and filtered to only contain transcripts that bore high similarity with those of *A. thaliana* transcripts and have a bit score of at least 80, the number of sequences had declined from starting 8000 sequences to 3000 sequences.

2.5.4.2 *Rationale behind aligning nucleotide sequences rather than amino acid sequences for annotation of basil's transcripts:*

For annotation of the basil's sequenced transcripts, the sequences were aligned against *A. thaliana* using nucleotide sequences rather than protein sequences. The aim of the alignment was to identify *A. thaliana* transcripts that bear very high similarity with

sequences of basil's transcript and identify different members from the families of genes. A DNA:DNA alignment is likely to show greater difference than protein: protein alignment, as a translated sequence is aligned per nucleotide but in amino acid sequence alignment, each amino acid is coded by different combinations of three nucleotides. Hence, any differences between sequences are likely to be picked up more in a nucleotide sequence than in amino acid sequence, allowing gene members from the same family to be more easily differentiated. In addition, very stringent conditions such as bit score of 80 and e-value of 10^{-14} were used when inferring homology between sequences, so that, no sequences were picked up that bore similarity but not a meaningful evolutionary relationship with each other. However, a major limitation associated with nucleotide sequence alignment approach is that DNA:DNA alignment has a much shorter evolutionary look back in time (5-10 folds) than protein:protein alignment[200]. Therefore, it may be possible that a lot of transcripts with significant similarity may have been filtered out that instead could have been picked up by carrying out a protein:protein alignment. Hence, it seems that in order to not loose significantly similar sequences as well as be able to differentiate members from gene families, it is important to carry out alignment of sequences in both formats, protein:protein as well as DNA:DNA.

2.5.4.3 Rationale behind the use of DAVID software for annotation of basil transcripts:

Genes are given identifiers and annotated with biological terms in databases such as NCBI and Entrez to aid researchers in making sense of biological information behind the gene expression data. However, due to the complex nature of biological research, the biological information of genes is spread out across different databases and maintained by independent groups[203]. Each database may have more than one identifier for the same gene and the same annotations associated with the gene identifiers may be found at different levels across databases[204]. Hence, in order to be able to interpret biological information from a large list of gene expression data, it becomes important to collate,

integrate and unify the information spread out across databases. The leading organisations such as NCBI and UniProt are largely redundant in nucleotide and protein sequences. Hence, NCBI and UniProt collated information independently either from databases such as EMBL, DDBJ, or Swiss-Prot, TrEMBL, and PIR respectively, to unify different types of gene or protein ID's and create non redundant databases of their own[203]. The resulting integrated data were added into newly formed databases called NCBI Entrez Gene, UniProt UniRef, and PIR-NREF. The integrated data limits the redundancy of the databases but lacked cross reference of ID's with other independent databases[203]. For instance, PIR ID are not referenced by NCBI and Refseq ID is not covered by UniProt. The weak cross reference of gene identifiers between NCBI and UniProt limits comprehensive integration across databases[204].

DAVID is the largest integrated database and provides a greater cross reference capability than either NCBI or UniProt. The DAVID knowledgebase is built around the DAVID single gene concept where 20 different types of gene identifiers obtained from different databases are agglomerated by a single linkage algorithm into a secondary gene cluster[204]. After integration the gene is given an integer that forms its own unique DAVID ID. The annotations are associated with their corresponding gene identifiers, hence, integration of different gene identifiers databases also agglomerates annotations and thereby enrich the gene with information from more than one annotation categories.

2.5.5 Identification of patterns through biased or non-biased clustering techniques:

Clustering techniques were used to analyse the changes in gene expression during and after the LR:FR treatment that was given between ZT4 and ZT8. Groups of genes conforming to expected patterns of gene expression were identified through biased clustering of genes using a pattern fitting regression method described by Devlin *et al*.[205] and provided in the appendix 2.2. The four samples compared were: HR:FR light at ZT6, LR:FR light at ZT6, HR:FR light at ZT10 and LR:FR light at ZT10, where all samples were

compared to their expression values to HR:FR light at ZT6. For obtaining an expected pattern, a template of a pattern was created in Microsoft Excel. The template involved designation of 0 representing low gene expression value and 1 representing a higher gene expression. The Pearson correlation coefficient of the expression pattern of each individual gene with the predicted profile was calculated (correlation depends upon slope and so is independent of absolute value). A P value was calculated for each correlation coefficient using a Student's t test to assign a level of confidence to the fit. Only genes with expression values across all four samples giving regression (R) values with 0.9 or higher and P values of 0.05 or less were selected as genes fitting a pattern. There were four expected patterns that were established. (1) genes that showed "permanent" upregulation, that is, they were higher in response to LR:FR light at ZT6 as well as following the LR:FR light treatment at ZT10 with a change in expression values of at least 1.5 fold or higher while showing no change in expression if maintained in HR:FR light between ZT6 and ZT10. (2) Genes that showed "permanent" downregulation, that is, they were lower in response to LR:FR light at ZT6 and at ZT10 by a gene expression fold difference of 0.66 or lower, while showing no change in expression if maintained in HR:FR light between ZT6 and ZT10. (3) Genes showing "temporary" upregulation in response to LR:FR light where gene expression fold difference was 1.5 fold or higher in LR:FR light at ZT6 but showed no change in gene expression observed following LR:FR light at ZT10. (4) Genes showing "temporary" downregulation where genes showed downregulation in response to LR:FR light at ZT6 by an expression fold difference of 0.66 or lower while no change in gene expression was observed in samples following LR:FR light at ZT10. The templates used for the four patterns of gene expression profiles for permanent upregulation, permanent downregulation, temporary upregulation and temporary downregulation were 0101, 1010, 0100 and 1011, respectively. Additional patterns that were not described as "expected patterns" were identified using the non biased K–means clustering tool of Mapman[206].

2.5.6 Gene ontology (GO) analysis of differentially regulated genes:

To analyse the gene ontology terms that are overrepresented (enriched), the Database for Annotation, Visualization and Integrated Discovery (DAVID) software[207] (https://david.ncifcrf.gov/tools.jsp) functional annotation tool was used with lists of genes that showed selected patterns of upregulation or downregulated. The three categories of gene ontology that were analysed included biological processes, cellular component and molecular function. Only the biological processes, cellular components and molecular functions with P values ≤ 0.05 were considered as reliable and discussed.

2.5.7 KEGG pathway analysis:

To investigate the biochemical pathways that were activated in response to changes in environmental stimuli, the DAVID functional annotation tool called "Pathway" was used to arrive at the pathway sub category called KEGG pathway where only biochemical pathways enriched among selected gene lists with significant P values were considered for analysis.

2.5.8 Mapman analysis of the basil transcriptome in response to chilling stress:

To analyse basil's metabolic response on exposure to chilling temperature, the 3000 transcripts with their AGI ID's along with their logged-to-the-base-of-2 differential expression values of control vs cold were uploaded into Mapman software[208] (http://mapman.gabipd.org/web/guest/mapman).

2.5.9 Determining reproducibility of first RNA sequencing experiment data

2.5.9.1 Rationale behind selection of FADS to determine reproducibility of RNA seq data.

FADS introduce double bonds (desaturate) in methyl chains of fatty acids and thereby increase membrane's resilience against rigidification by chilling temperatures. The effects of knock out FAD mutants and their overexpression on plants tolerance to chilling conditions has been described in chapter 1. A positive correlation between the degree of membrane desaturation and chilling tolerance was found in transgenic tobacco and cyanobacteria[209]. In addition, an upregulation of FADS by low temperature was found in *A. thaliana* and maize[210–212]. FADS are therefore considered to play important role in the development of plants tolerance to chilling temperatures and were therefore considered genes of interest. Five members of FADS family were sequenced, successfully identified as homologous to *A. thaliana* and showed similar expression to each other. Thus, all five members including FAD2, FAD5, FAD6, FAD7, and FAD8 transcripts were used for measurement of differential gene expression measurement by qCPR to test the reproducibility of RNA seq data.

2.5.9.2 Statistical analysis of qPCR results for FADS.

The differential gene expression of FADS was calculated using the Livak or delta delta Ct method. Three biological replicates and two technical replicates for each sample were used when calculating differential gene expression. Initially, three means were calculated for each sample using two technical replicates for each biological replicate and standard deviation (square root of the variance) was calculated from the three means. The standard error was calculated using the standard deviation of the three means and dividing the standard deviation by n = 3. The melt curves and the PCR amplified sequences matching the regions amplified by qPCR are provided in appendix 2.3, 2.4 and 2.5.

2.6 Measurement of differential gene expression:

2.6.1 Primer design of genes of interest:

MetE primers were designed by simply uploading its nucleotide sequence onto Primer3 software[213,214] (http://bioinfo.ut.ee/primer3/), but the remaining other primers were designed using the method described below.

When designing primers for genes of interest, the aim was to avoid the conserved domain and the ion binding domain whenever possible and use 5' or 3' UTR regions instead, as they offer greater specificity towards binding of the primers onto the target sequence. The protocol used for designing primers for each gene of interest involved the use of amino acid sequence as well as nucleotide sequence from basil and *A. thaliana.* The amino acid and nucleotide sequences were used to carry out an amino acid and nucleotide sequence alignment between basil and *A. thaliana* genes to assess the regions of homology, which were subsequently used when designing the primers. The amino acid sequence of genes from basil were aligned using ClustalW[215] (http://www.ebi.ac.uk/Tools/msa/clustalo/) against an amino acid sequence of the same gene from *A. thaliana.* If there was high homology, then the conserved domain and the ion binding domain were obtained from the NCBI protein database graphic tool for amino acid gene sequence of *A. thaliana.* In the nucleotide sequence of basil's gene, parts of its nucleotide sequence that were homologous to the conserved and the ion binding domain of *A. thaliana's* gene were identified and avoided when designing primers. Only the parts of the sequence that were not part of a conserved domain or the ion binding domain but were still homologous to *A. thaliana* gene sequence were selected for primer design (wherever possible). The primers were designed by uploading the selected part of the basil's nucleotide sequence onto the Primer 3 software. From the range of primer options produced, only those with the length

of 19-22 bp, melting temperature Tm of 58^0C-62^0C, GC content of 40-60% and yielding product size of 100-150bp were selected for measurement of differential gene expression.

2.6.2 Synthesis of cDNA and qPCR

1 ug of RNA samples were used to synthesise cDNA using the instructions provided in QuantiTect Reverse Transcription Kit (Qiagen, UK). The extracted total RNA samples were treated with gDNA Wipeout buffer before being transcribed into cDNA. The gDNA Wipeout buffer was added to each RNA sample to ensure that the RNA samples were eliminated and free from any potential gDNA contaminants[216]. The gDNA wipe out buffer during cDNA synthesis was also used by Dr Sandra Smeiszek when preparing RNA samples for RNA sequencing. Hence, the cDNA samples used for RNA sequencing and qPCR experiments were likely to be free from gDNA contaminants.

Samples were prepared for qPCR using cDNA, primers, SYBR Green JumpStart Taq ReadyMix (Qiagen, UK) and RNase-free water (Qiagen, UK) using a qiagility robot (Qiagen, UK). The prepared samples with a total volume of 20µl were then loaded into a Rotor Gene –Q (Qiagen, UK) for qPCR.

2.6.2.1 Analysis of the differential gene expression values of genes by the Livak or delta delta Ct method using Ct values obtained from qPCR.

The data obtained from qPCR (Ct values) were analysed by a relative quantification method using the Livak or ΔΔCt method that assumes the efficiency of the reaction to be 100%. Using this method the Ct values of the gene from control and the treated sample were firstly normalised to the reference gene to avoid the need for accurate quantification of the starting material and were normalised in the following manner:

In this present study, depending on the experiment, the control condition and the treatment condition was either ambient temperature and cold temperature, respectively,

or HR:FR light ambient temperature as control and treatment conditions as LR:FR ambient temperature, HR:FR cold temperature and LR:FR cold temperature

ΔCt control sample = Ct (target gene, control) – Ct (reference gene, control)

ΔCt treated sample = Ct (target gene, treatment) – Ct (reference gene, treatment)

The change in the expression of the gene of interest between the test and the control and normalisation of any differences in the loading of the sample between the reference gene and the treatment gene, the ΔΔCt was calculated in the following manner:

ΔΔCt = ΔCt (target gene, treatment) – ΔCt (target gene, control)

The differential gene expression, assuming that the amplification of the target gene was doubled by each cycle, was calculated as fold difference as

$$\text{Differential gene expression} = 2^{-(\Delta Ct\ \text{Control} - \Delta\Delta Ct\ \text{target gene})}$$

Having calculated the differential gene expression data for aquaporins, the following normalisation procedure was carried out between biological replicate experiments prior to plotting the mean values. For each replicate experiment, relative expression values across the experiment were all normalized so that the HR:FR samples showed the same relative expression scale over the time course in each case. For each aquaporin, the scale was normalised to the mean maximum expression value for basil plants treated with HR:FR ambient conditions over the time course.

2.6.2.1.1 PCR purification of amplified genes:

The products of qRT-PCR were frozen at -80^{0}C for subsequent purification to be sent for gene amplified product sequencing. The PCR purification of the qRT-PCR was carried out according to the protocol provided by the QIAquick PCR Purification Kit (Qiagen., UK). 5µl of the purified product was run on a 1.5% agarose gel to establish whether the purified product created a band on the gel and the size of the band corresponded to the product size of the amplified transcript. On seeing the band and cross checking the size, the

products were diluted according to the instructions of the Eurofins sequencing company (Eurofins Genomics, Germany). The returned sequence data were compared with the primers and sequence isolated to determine whether the gene was, in fact, the gene that was amplified.

3 CHARACTERISATION OF THE RESPONSE OF BASIL TO CHILLING TEMPERATURES

3.1 Introduction:

Numerous physiological disturbances occur in plants when treated with chilling temperatures that can lead to the development of chilling injury in tropical and subtropical plants[87]. The chilling injury symptoms (leaf wilting and discolouration) can cause the appearance of the crops to become unappealing to the consumer's eye and have a detrimental effect on their sales.

In this chapter, the effect of exposure to chilling temperatures was investigated on the basil variety Marian. Basil variety Marian is grown and supplied to numerous UK supermarkets by Vitacress. Basil has previously been reported to be sensitive to chilling temperatures[217,218] and the deterioration in the quality of basil plants was suggested to have been observed only after its exposure to chilling conditions; this can occur during its transportation from the site of production to those of the buyers or at the retailers (Budge pers comm). Hence, it was important to investigate the effect of chilling temperature on this variety Marian that is grown in Vitacress' own glasshouses and to establish methods that could be used to assess damage and its alleviation by innovative treatments.

Given that transport from the grower to the retailer can take 24 - 72h and for this time plants will be held in the dark the response of basil to 24 or 48 h of chilling temperature (4^{o}C) under continuous darkness was investigated using physiological assays that assess the intactness of cellular components commonly affected by chilling temperature in chilling sensitive plants.

3.1.1 Assesment of chilling induced cell membrane damage by electrolyte leakage assay:

Cell membrane damage in response to the low temperature of the environment is commonly measured by measuring electrolyte leakage [186,219–222]. Cellular membranes compartmentalise electrolytes in the living cells. Under stress, or during senescence, the

lipids or proteins of the membrane get damaged and this can cause an increase in the membrane's permeability[186,223]. Damaged cellular membranes with a loss of integrity result in electrolytes leaking out from the cell into the surrounding apoplast whereas electrolytes in healthy cells with intact membranes remain compartmentalised[224]. The conductivity of the bathing solution of damaged or healthy cells can be measured, and a high electrolyte leakage value is assumed to reflect greater membrane damage.

Increased electrolyte leakage of non-acclimated *A. thaliana* occurred after freezing at -2^{0}C and reached its maximum magnitude of 95% leakage at -5^{0}C. However, after cold acclimation of 1 d, the increase in the electrolyte leakage from leaves did not occur until after freezing at -4^{0}C[96]. The electrolyte leakage assay also showed that after 1 d of cold acclimation 50% of the electrolytes from *A. thaliana* leaves (T_{EL50}) were diffused into the solution at -7^{0}C[96]. After 7 d of cold acclimation, the maximum freezing tolerance in *A. thaliana* leaves was achieved where T_{EL50} was reached at -10^{0}C. There was not any further increase in freezing tolerance in *A. thaliana* leaves following 14 d of cold acclimation. There were other parameters that were used as determinants of freezing injury in *A. thaliana* protoplasts. Hexagonal II phase formations were observed in the electron micrographs of *A. thaliana* protoplasts isolated from non-acclimated seedlings at -6^{0}C. However, there was no hexagonal II phase observed in protoplast isolated from 7 d acclimated seedlings. These findings are in agreement with the findings of electrolyte leakage assay, as they showed that non-acclimated seedlings of *A. thaliana* experienced greater freezing injury than seedlings acclimated for 7 d. This demonstrates that electrolyte leakage assay is an effective assay for determining cold injury in freezing sensitive tissues of *A. thaliana.*

3.1.2 Measurement of ROS accumulation in basil leaves as a marker of chilling injury:

According to Kane *et al.*[225] mechanisms aside from membrane rigidification such as oxidative stress must also contribute to the development of chilling injury in plants, as some plants are more sensitive than others even though they possess membranes with a

similar lipid composition. Under normal conditions, ROS are produced at a low level in the chloroplast, mitochondrion and peroxisomes. However, during stress, their production is dramatically elevated[226]. The increased ROS production during environmental stress can play two contrasting roles; stress protection or stress injury. ROS may be involved in temperature stress perception by inducing expression of stress responsive genes or injure plant tissues by oxidising cell membrane lipids and proteins[227]. ROS was shown to have increased in its content and caused damage to cells of *A. thaliana* and seedlings of maize when transferred from 23^0C or 27^0C to 4^0C[225,228]. Einset *et al*.[229] found that the ROS accumulation caused inhibition of root growth upon transfer of plants to ambient temperatures. However, when the ROS accumulation was prevented by treatment of the plants with glycine betaine, then the root growth resumed upon transfer of the plants to ambient temperatures. These studies show the role of ROS in the development of chilling injury and thereby suggest a link between ROS accumulation and chilling injury. Hence, considering the evidence and the suggestion of the link between elevated ROS levels during times of chilling stress in sensitive plants, changes in the content of ROS in basil leaves were measured as a marker of chilling injury to determine if it was sensitive to chilling temperatures.

3.1.3 Excessive generation of ROS can lead to lipid peroxidation of cells.

Oxidative stress generated during chilling stress can activate lipid peroxidation of cells in chilling sensitive plants[186,230,231]. Lipid peroxidation is a process involving a reaction between a free radical and polyunsaturated fatty acid molecule of the cell membrane[232]. In the process of lipid peroxidation, the free radical attacks the carbon to carbon double bond and causes a removal of hydrogen. This oxidation of polyunsaturated fatty acids can decrease the number of unsaturated fatty acids in the membrane, reduce membrane fluidity and increase permeability[233].

The process of lipid peroxidation is described by Ayala *et al*.[234] in three steps that include initiation, propagation and termination. In the initiation step, the process of lipid peroxidation commences from the pro-oxidant hydroxyl radical reacting with polyunsaturated phospholipid and removing hydrogen from the carbon of the acyl chain to generate a lipid radical (L●) (Figure 3.1). In the propagation step, the resulting lipid radical can react with oxygen to form lipid peroxyl radical (LOO●) (Figure 3.1). The (LOO●) radical then reacts with yet another polyunsaturated phospholipid to attain a hydrogen and form lipid peroxide (LOOH) (Figure 3.1). This reaction results in the generation of another (L●). The reaction continues to take place and increases the number of lipid radicals produced until an antioxidant (vitamin E) donates a hydrogen atom to the (LOO●) species and terminates the reaction [235] (Figure 3.1).

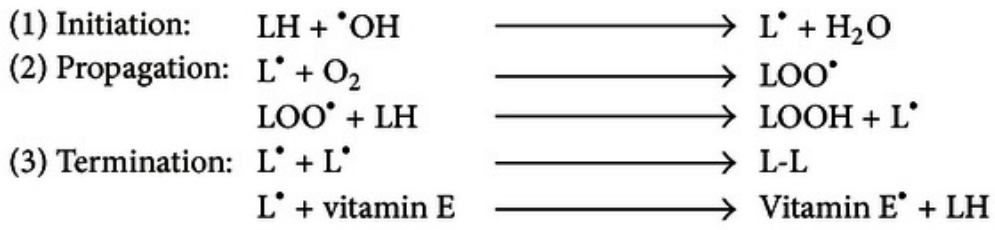


Figure 3.1 The process of lipid peroxidation from initiation, propagation to termination. The process involves generation of lipid radicals (L●) that in turn can react with other lipid molecules or oxygen molecule and result in production of more lipid radicals (LOO●) Reproduced from Yadav and Ramana[236].

Lipid peroxidation in cells is an indicator of oxidative stress and can cause inhibition of membrane functions, increased electrolyte leakage, destruction of the barrier properties of the membrane and through increased cytosolic Ca^{2+} concentration can even lead to cell death [237].

Lipid peroxidation results in the generation of many aldehydes as its secondary products. One of the aldehydes that has been studied further and used as a reliable marker to measure the degree of lipid peroxidation in tissues is malondealdehyde (MDA)

compound[234]. The content of MDA being generated from the process of lipid peroxidation was therefore used as a measure of oxidative stress in the present study.

3.1.4 Effect of chilling temperature on antioxidants defence system of plants:

Activity of antioxidant defence systems (enzymatic or non-enzymatic) in plants are increased in response to increased generation of oxidative stress at times of abiotic or biotic stress conditions[238]. For instance, *A. thaliana* cells and maize seedlings that showed an increase in oxidative stress under chilling conditions also showed an increase in ascorbate peroxidase activity or content of guaiacol peroxidase and catalase 3, respectively[225,228].

Chilling injury symptoms have been established to be linked with the accumulation of ROS species[226]. Thus tolerance from chilling induced damaging effects have been found related to enhanced activities of antioxidative enzymes and higher activities of antioxidative enzymes are correlated with higher stress tolerance[220,231,239]. For instance, a chilling tolerant variety of rice showed a much higher antioxidant activity than the chilling sensitive variety[220]. Furthermore, an increase in the polyamine content was observed to occur in the chilling tolerant variety of cucumber under chilling conditions but not in the chilling sensitive variety[132]. Therefore, the content of water soluble antioxidants content was measured in basil, in order to investigate if plants had the ability to increase the activity of antioxidant defence systems to protect itself from chilling induced oxidative stress.

3.1.5 Effect of chilling temperature on photosynthesis and chlorophyll fluorescence of plants

One of the oxidative damages experienced by chilling sensitive plants at chilling temperatures is the degradation of chlorophyll molecules and carotenoids leading to reduced efficiency of photosynthesis[240–242]. For instance, three tolerant and three sensitive maize genotypes were treated at chilling temperatures, and all three chilling

tolerant genotypes showed a higher photosynthetic activity, higher content of chlorophyll a + b, higher chlorophyll a/b ratio and larger carotenoid pool size than the chilling sensitive genotypes[241].

Under illumination, the excess light energy not used by photosynthesis in plants is dissipated either as heat or re-emitted as fluorescence also known as prompt fluorescence or chlorophyll fluorescence. In 1951, Strehler and Arnold[243] discovered a second type of emission that could last longer than the prompt fluorescence after switching off the light. This type of fluorescence is called delayed fluorescence and occurs as a result of a dark back reaction where the light excitation energy is transferred from Q- (primary PSII electron acceptor) to Z+ (primary PSII electron donor)[244].

On treatment with chilling temperatures, chilling sensitive plants showed reduced yield of chlorophyll fluorescence or delayed fluorescence due to them having experienced chilling injury in the chloroplast membranes and loss of chlorophyll molecules[240,245–247]. Hence, measurements of chlorophyll florescence or delayed fluorescence have been used in the past as screening techniques for determining the sensitivity of plants to chilling temperatures[240,244]. Similarly, in this chapter a crude assay of delayed fluorescence was used to investigate the effect of chilling temperature on the content of chlorophyll molecules in basil seedlings treated with or without chilling temperatures.

Changes in the state of the membrane, the content of MDA, accumulation of ROS, activity or the content of antioxidants or in the content of the chlorophyll molecules were often observed in plants sensitive to chilling temperatures[186,225,228,246]. Hence, physiological assays that are sensitive to changes in the cellular sites and cellular molecules described above were used to investigate the effect of chilling temperature on basil leaves cellular components. The results obtained are used to elucidate if basil is sensitive to chilling temperature and if its treatment during transportation at 4^0C for 24 or 48 h is likely to be problematical for either its sales or wastage.

3.2 Results and Discussion:

The aim of the chapter was to determine the sensitivity of basil towards chilling temperatures. The response of basil towards chilling temperatures was investigated by assessing its cellular components intactness under chilling stress for 24 h. The assessment of damage to basil cellular components post chilling stress was determined using the most widely accepted methods of chilling sensitivity markers such as electrolyte leakage assay, measurement of ROS by DAB assay, lipid peroxidation using TBA assay, delayed chlorophyll fluorescence and measurement of antioxidants accumulation by FRAP assay.

To establish the duration of exposure to chilling temperature that leads to the development of chilling induced damage on basil tissues, at the visual level, basil plants were exposed to varying duration of chilling temperatures from 0 h, 24 h and 48 h (Figure 3.3 a – g).

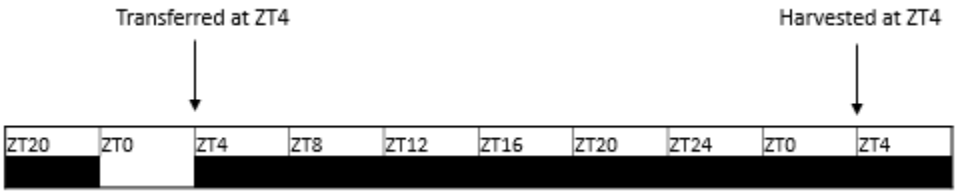


Figure 3.2 Plants were brought from Vitacress, and the next day, plants were transferred into either ambient or chilling temperatures for 24 h under continuous darkness. Figure above shows the time points when basil plants were transferred into cold or ambient conditions (ZT4) and harvested the next day (ZT4). The black and white colour in the diagram represents the duration of dark and light conditions the plants were placed in, respectively.

(a) 0 h (b) 12 h

(c) 24 h d) 48 h

(e) 0 h (f) 24 h (g) 48 h

Figure 3.3 Images of basil exposed to varying durations of chilling temperature (4^{o}C). The images are representative of 10 pots per time point. (a, b, c and d) from the side (e, f and g) from above.

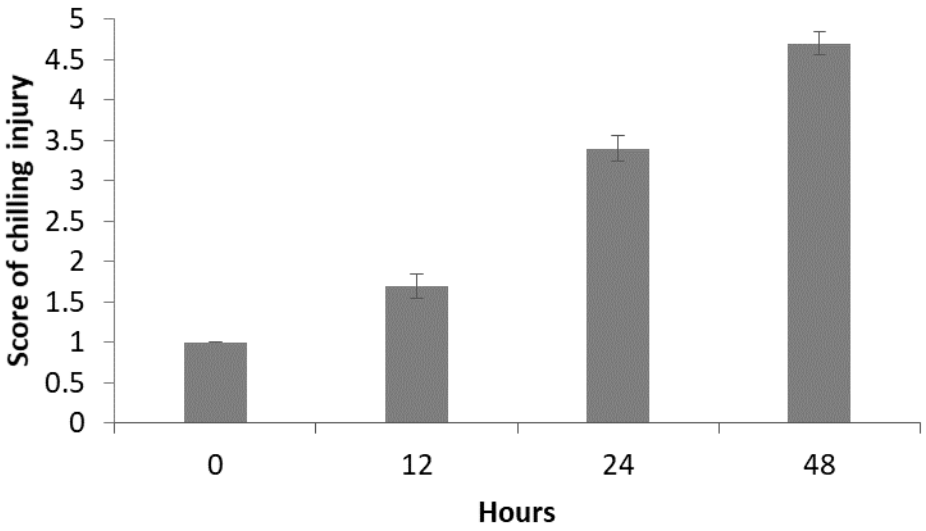


Figure 3.4 Basil pots were exposed to varying durations of chilling temperature 4^0C varying from 0 h to 48 h, photographed and scored according to the degree of damage the pots of basil displayed following their duration of exposure to cold temperature. Each value represents the mean ± SE and n = 10.

The grading of the basil pots exposed to chilling temperature ranged on the scale of 1 to 5, where 1 represented no damage, 2 relates to basil exhibiting slightly wilted leaves, 3 relates to those pots with heavily wilted leaves, 4 wilted leaves and discoloration on less than 50% of the leaves and 5 represents wilted leaves with discoloration on more than 50% of the leaves in the pot. The differences observed in basil's response to four different chilling temperatures (Figure 3.3) (0 h Vs 12 h, 0 h Vs 24 h, 0 h Vs 48 h) were found to be statistically significant according to one way ANOVA and post-hoc Tukey's HSD test at $P < .05$ where P value was $1E^{-05}$ and 0.0046, 0.001, 0.001 and 0.001, respectively.

The symptoms of chilling injury on basil pots became visually apparent from 12 h after exposure to chilling temperatures. Using the grading criteria described above basil appeared on average more damaged after 12 h of chilling treatment, due to the slight wilting of the leaves as compared to the basil pot not exposed to chilling temperatures (Figures 3.3a, 3.3b and 3.4). However, a visually significant difference was observed after treatment of basil pots with cold temperatures of 24 h where the damage experienced was

greater than 12 h chilling treated basil (Figures 3.3c, 3.3f and 3.4). The basil pots condition continued to deteriorate and by 48 h the deterioration in the quality of the pot was worse and the plant appeared dead compared to control basil pots (Figures 3.3d, 3.3g and 3.4). Moreover, the transportation of the herbs from the site of the growers to that of the retailers distribution centre takes 24-48 h (Budge pers comm.) Figures 3.3 a-g show that the wilting of the leaves continued up to 48 h indicating that leaves of basil on continued exposure to chilling temperatures causes the leaves to continue to loose water and wilt. However, 24 h of chilling temperatures was used as a standard duration for physiological and molecular analysis to be carried out with as this is likely to be the minimum time that plants could be exposed to chilling temperatures and the situation is likely to worsen if the exposure time increases.

3.2.1 Fresh weight:

On exposure to chilling temperature, many plants experience loss of water due to an imbalance in the root water uptake and the water lost from the leaves[248]. Hence, the fresh weight of leaves was measured after exposure to 24 h of cold temperatures.

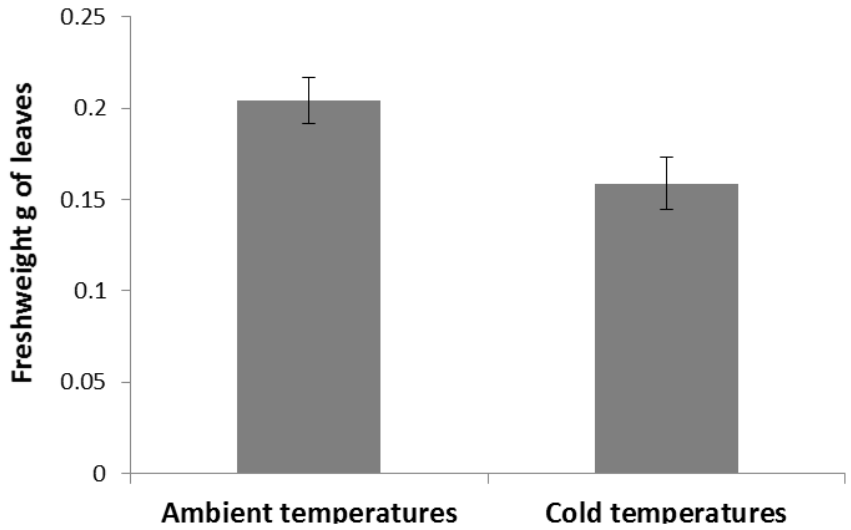


Figure 3.5 The fresh weight (g) of individual leaves of 29 days old basil exposed to either 24 h of dark and cold or dark and ambient conditions. Each value represents mean ± SE and n = 10.

The chilling treatment resulted in loss of fresh weight (Figure 3.5) by nearly 25%, supporting the hypothesis and providing a further measure of chilling damage in basil.

3.2.2 Electrolyte leakage

Electrolyte leakage assays were carried out in different ways in different studies[186,187,249]. Hence, in this present study the electrolyte leakage assay used by Campos *et al*.[186] and Bajji *et al*.[187] was adopted and validated before using it for determination of the sensitivity of basil towards chilling temperatures. Below are series of experiments aimed at determining the optimal protocol for the electrolyte leakage assay.

3.2.2.1 Optimisation of the electrolyte leakage assay for the estimation of the cell membrane stability in basil leaves.

In order to determine the effectiveness of the electrolyte leakage assay designed using methods described in studies by Campos *et al*.[186] and Bajji *et al*[187], basil tissues were exposed to chilling temperature for a longer duration i.e. 48 h that nearly always resulted in leaf breakdown and development of leaf discoloration. The control (healthy) and chilling treated (damage) tissues were subjected to the electrolyte leakage assay (material and methods, electrolyte leakage assay – version 1) and the results analysed to assess the difference in the electrolyte leakage values measured.

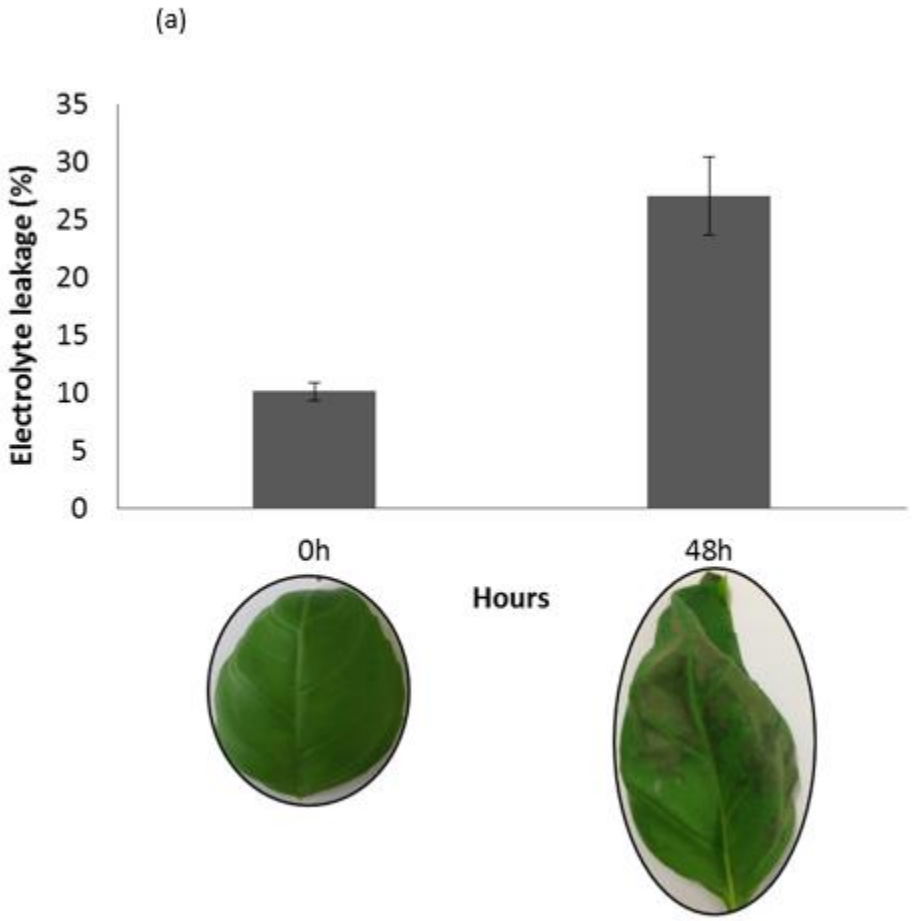


Figure 3.6 (a) Electrolyte leakage (% of total electrolytes) from whole leaves of 29 days old basil suspended in water for 12 h. Each bar represents mean ± SE and n = 10. Below the graph are the images of leaves exposed to either 0 h or 48 h respectively showing representative leaves.

Basil plants exposed to 48 h of cold showed significantly increased electrolyte leakage (T-test at a P value of 0.002) from the leaves not treated by cold temperature (Figure 3.6a). The results showed that the assay was suitable and effective in measuring chilling injury. Having established a protocol effective in measuring leakage of electrolytes from basil leaves, the same assay was used to investigate if the time consumed by the protocol could be reduced. The electrolyte leakage values were, therefore, measured every 5 minutes for 2 h from basil leaves treated with and without 24 h of chilling temperature (Figure 3.7).

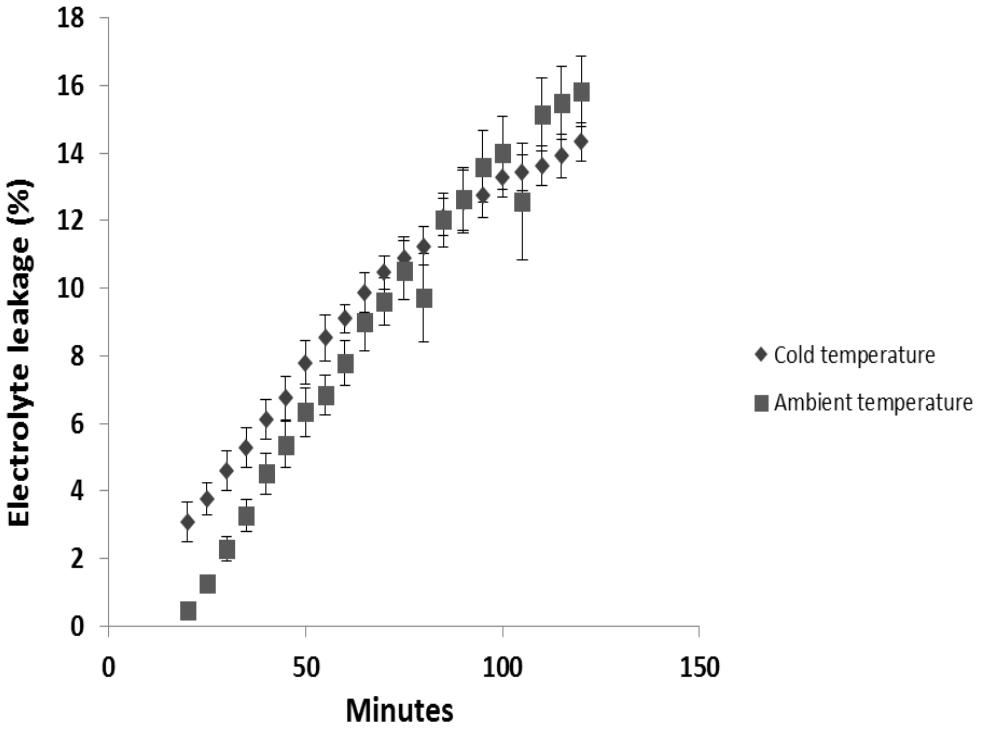


Figure 3.7 Determination of membrane damage using electrolyte leakage from leaves of cold-treated plants or those held at 22^{0}C. Electrolyte leakage measured every 10 minutes and compared to the final conductivity of the bather solution after autoclaving the leaves. Each bar represents mean ± SE and n = 5.

Results from this protocol did not show a large difference between control and cold treated basil leaves (Figure 3.7). Therefore instead of measuring the electrolyte leakage values over minutes the electrical conductivity was measured over hours (Figure 3.8).

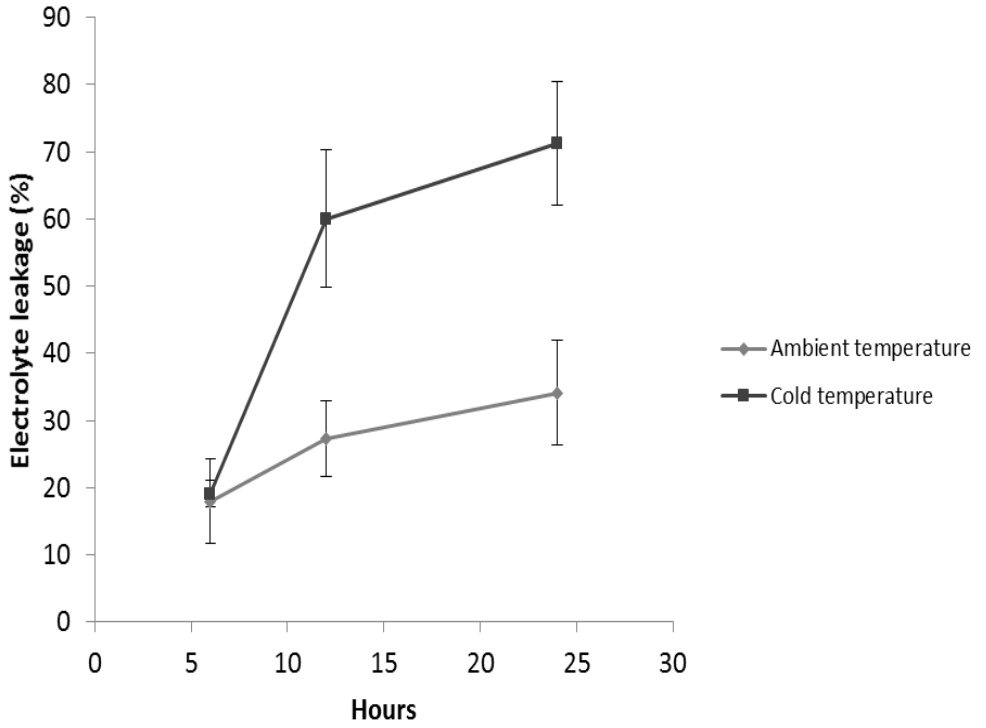


Figure 3.8 Electrolyte leakage (%) measured from true (top) leaves of basil exposed to either 24 h of continuous darkness and ambient temperature 22^{0}C. Each data point represents mean ± SE and n = 5

On measuring the electrolyte leakage values at different time points of every few hours (6 h, 12 h and 24 h), the greatest difference between samples treated with or without chilling temperatures was observed at the 24 h and 12 h time point, respectively (Figure 3.8). However, it was the 12 h time point that was chosen to be used as a final electrolyte leakage value reading in the next electrolyte leakage experiments as using 12 h time point rather than 24 h shortened the time consumed by the protocol by 12 h.

Whilst differences in membrane permeability were detected in the uppermost leaves after exposure to chilling temperature (Figure 3.8) such differences were not apparent in the lowermost leaves (Figure 3.9)

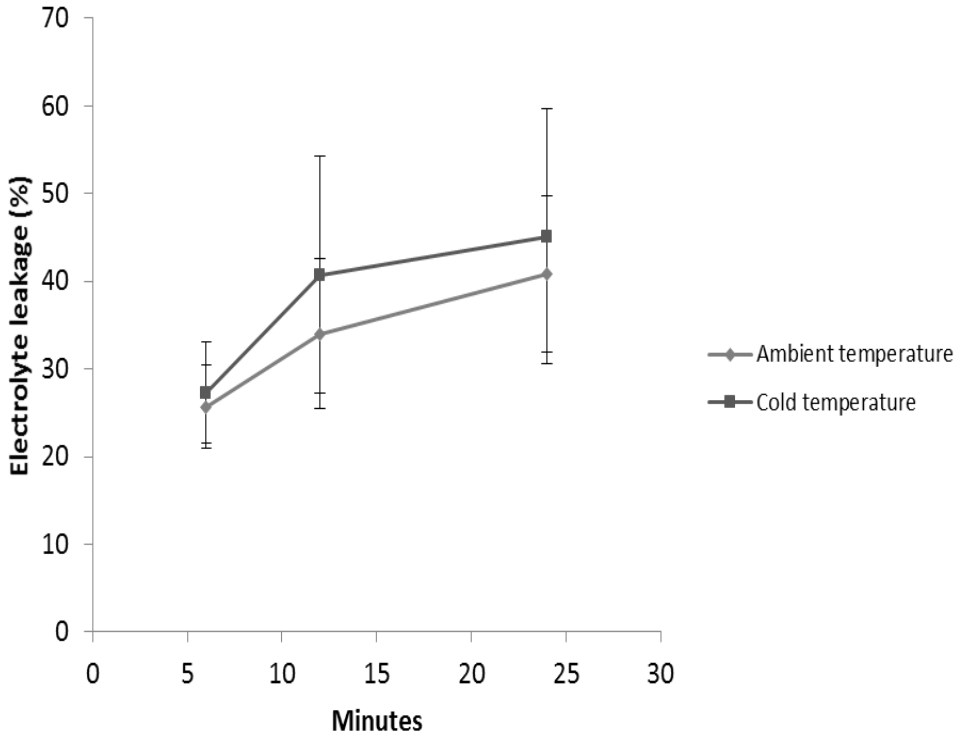


Figure 3.9 Electrolyte leakage (%) measured from bottom leaves of basil exposed to either 24 h of continuous darkness and ambient temperature 22^{0}C or 24 h of continuous darkness and cold temperature 4^{0}C. Each data point represents mean ± SE and n = 5.

The results obtained from electrolyte leakage of the bottom leaves show that the extent of electrolyte leakage differed considerably between top and bottom leaves (Figure 3.8 ad 3.9). This could be due to the bottom leaves being insulated by the upper leaves and thereby resulting in them showing reduced electrolyte leakage (i.e. more intact membranes). The electrolyte leakage values from the bottom leaves suggest that positioning of the leaves on basil plants are important and to be considered when determining the sensitivity of the plant to chilling temperatures. As the difference in leakage of electrolytes was observed to be greater in top leaves than bottom leaves, all subsequent measurements were taken using the uppermost fully expanded leaves.

The optimised protocol established for measuring the leakage of electrolytes from the basil tissues is described in method electrolyte leakage assay – version 2 (Figure 3.9).

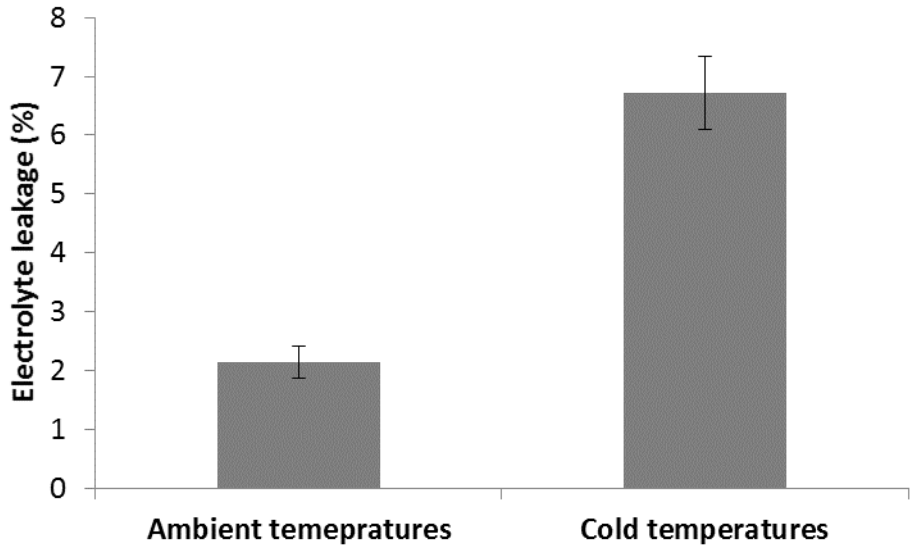


Figure 3.10 Electrolyte leakage values (%) of entire basil's leaves exposed to either continuous 24 h of dark and cold (4^0C) or dark and ambient (22^0C) conditions. Each value represents the mean ± SE where n = 5. Font

Leaves with cells that have intact membranes leak fewer ions and will, therefore, give low conductivity values compared to leaves with damaged cell membranes. Results from the electrolyte leakage assay show that basil tissues exposed to 24 h of chilling temperature leaked electrolytes significantly more and by 2.15 fold that from basil plants treated at ambient temperatures (Figure 3.10, T test derived p value = 4.45×10^{-5}). Hence, the results obtained from the electrolyte leakage assay indicate that the cell membranes of basil leaves are likely to have experience damage when plants were stored at 4^0C as compared to storing at 22^oC.

The electrolyte leakage test is simple, generates quantitative data and is a rapid way of determining the susceptibility of the plants to chilling temperatures [250]. However, the electrolyte leakage assay alone was not sufficient to evaluate membrane damage, other parameters including lipid peroxidation and ROS measurements were therefore used to determine basil's response to chilling temperatures.

3.2.3 ROS accumulation in leaves

The principle of brown colour appearing on the leaves was exploited to detect whether basil, when exposed to chilling temperature, experiences stress and thereby increases in ROS content in its leaves. The ROS assay was carried out using the top most fully expanded leaves.

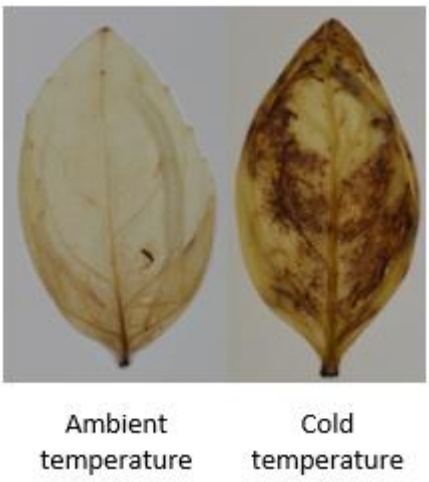


Figure 3.11 Measurement of ROS accumulation using the DAB assay on 29 days old basil leaves whose plants were either treated with 24 h of continuous darkness and ambient temperature (22^0C) or darkness and cold temperature (4^0C). Each bar represents mean ± SE and n = 15. Dark areas indicate the presence of H_2O_3 as these are the areas where DAB was oxidised by H_2O_3 in the presence of heam containing enzyme.

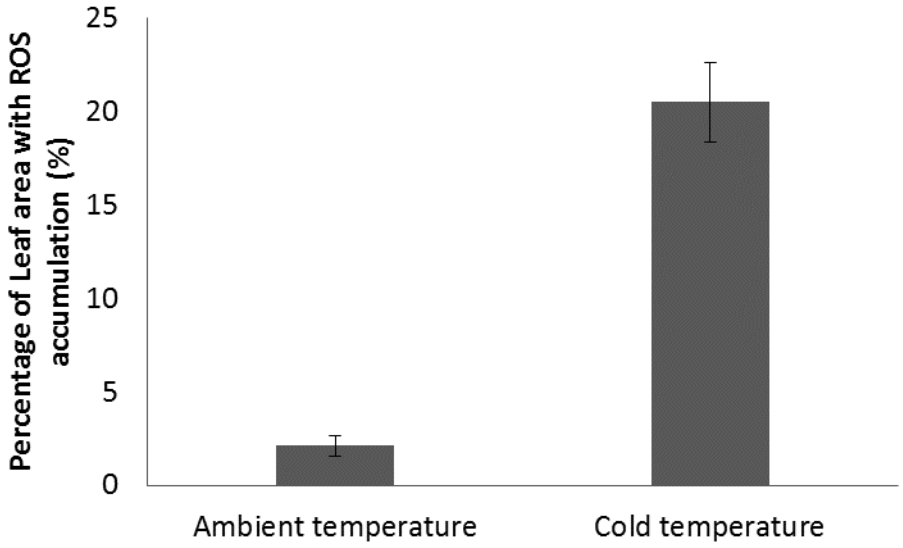


Figure 3.12 Percentage of leaf area containing ROS, in leaves of basil exposed to either 24 h of continuous darkness and ambient temperature (22^{0}C) or darkness and cold temperature (4^{0}C) measured using DAB assay. Each bar represents mean ± SE n = 15.

The amount of leaf area in which ROS accumulated was significantly higher in basil leaves treated with chilling temperatures compared to basil treated at ambient temperatures by approximately nine fold (Figures 3.11 and 3.12, T test, p value = 3.19417 x 10^{-07}). The difference in the accumulation of ROS at chilling temperature, clearly indicates that the generation of ROS species is dramatically increased upon treatment of basil plants at chilling temperature and is likely to have caused oxidative damaging effects on cellular components of basil plants.

3.2.4 Lipid peroxidation

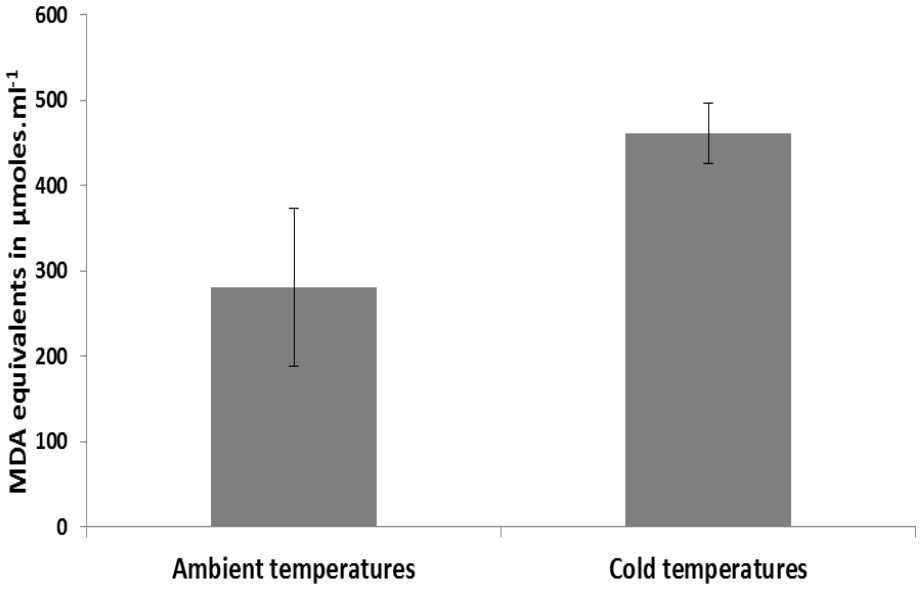


Figure 3.13 MDA equivalents measured from leaves of 29 days old basil treated with or without chilling temperature 4^0C under 24 h of continuous darkness. Each value represents the mean ± SE and n = 5.

The extent of lipid peroxidation in tissues was indicated by measurement of the lipid peroxidation secondary product malondialdehyde (MDA). The measurement of MDA content in basil tissues showed that the process of lipid peroxidation took place to a greater extent in the chilling treated basil compared to basil maintained at ambient temperatures (Figure 3.13). However, the increase in the MDA content of chilled basil leaves was not found statistically significant. The higher accumulation of MDA content suggests that the membrane integrity of chilled basil may have been comprised to a greater extent than basil maintained at ambient temperature. However, this conclusion can only be reached with further experimentation.

High levels of lipid peroxidation in basil leaves were believed to be due to increased accumulation of ROS in chilling treated basil tissues (Figure 3.11, 3.12 and 3.13). The increased oxidative stress probably preferentially reacts with the polyunsaturated phospholipids in the membranes of the cell and its organelles, resulting in lipid degradation

and the accumulation of products such as MDA and lipid hydroxyl radicals[234]. Basil leaves cellular membranes showing greater membrane lipid peroxidation are also likely to have contributed to the increased electrolyte leakage values described above upon basil's treatment at chilling temperature compared to basil treated with ambient temperatures.

3.2.5 Antioxidants content

The protective mechanisms (water soluble antioxidants) produced by the cell to avoid damage from endogenously generated oxidants was measured.

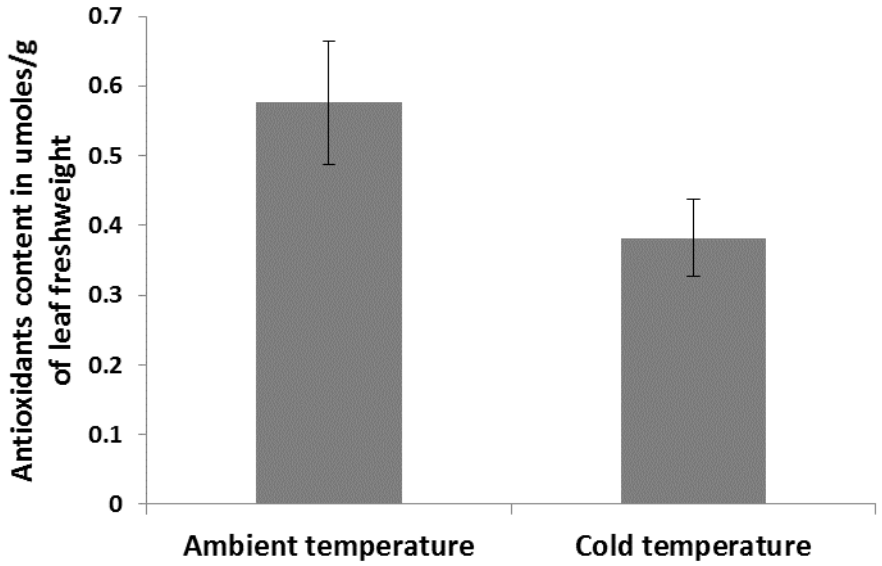


Figure 3.14 Water soluble antioxidants content equivalent to that of ascorbic acid of entire 29 days old basil leaves exposed to either 24 h of darkness and cold or darkness and ambient temperature. Each value represents mean ± SE (n = 10).

It appears that 24 h of chilling temperature exposure to basil leads to a statistically significant decline in the water soluble antioxidant content by 31% compared to basil treated at ambient temperatures (Figure 3.14, T test derived P value = 0.0037). The reduced content of water soluble antioxidants at times of chilling stress in basil leaves suggests that basil may possibly lack the ability to induce protective mechanisms against

the generation of ROS. The reduced content of water soluble antioxidants could, therefore, be one of the possible reasons that may have contributed to the increased accumulation of ROS in basil leaves during chilling stress.

3.2.6 Delayed chlorophyll fluorescence

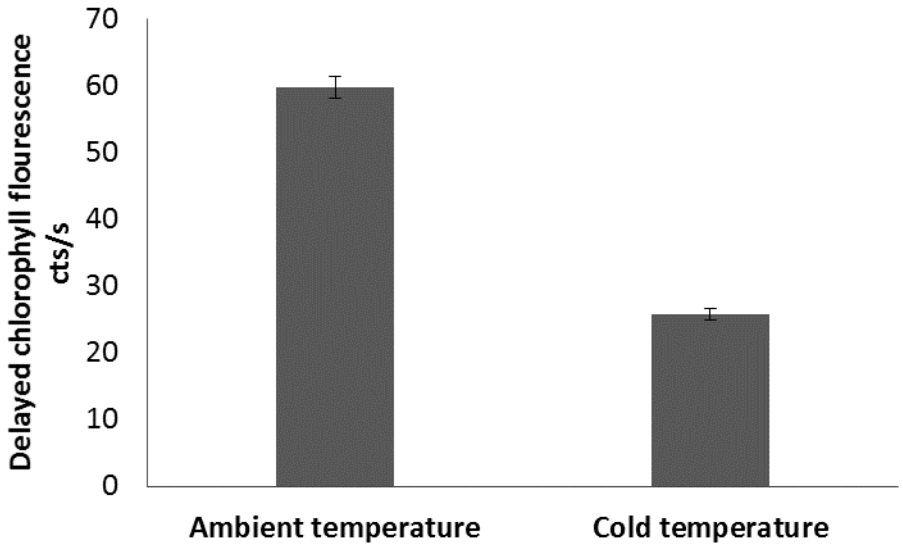


Figure 3.15 Delayed chlorophyll fluorescence from 10-day old basil seedlings treated with or without chilling temperature for 24 h under continuous darkness. Each value represents the mean ± SE (n = 12).

Measurement of delayed chlorophyll fluorescence from basil seedlings, showed that the chilling treatment led to a statistically significant decline in the yield of chlorophyll fluorescence by 32 cts/s (Figure 3.15, T test P value = 2.30175×10^{-14}). This represents a significant decline the content of chlorophyll molecule and suggests for basil's chloroplast and its constituents to have likely experienced structural damage as a result of exposure to chilling temperature.

Altogether the physiological assays, including electrolyte leakage assay, lipid peroxidation, DAB assay, FRAP assay and delayed chlorophyll fluorescence, showed an increase in membrane permeability, ROS accumulation and phospholipids degradation by 2.15, 9 and 0.64 fold respectively (Figures 3.10, 3.12 and 3.13) and decrease in antioxidants and chlorophyll content by nearly 50% (0.56 and 0.57 fold respectively) (Figures 3.14 and 3.15).

At times of membrane phase change in the cellular membranes of plants during chilling stress, the structural defects occur leading to enhanced permeability in a less fluid domain[251]. The increase in the permeability of the basil cellular membrane observed after treatment with chilling temperatures indicates that the biophysical properties of its membranes have possibly undergone a phase transition from liquid to solid gel-like state. Increases in electrolyte leakage from the membranes could also develop as a result of damage to membrane components by lipid peroxidation induced by the chilling conditions[186]. The sensitivity of plants towards chilling temperatures (as seen visually) and the electrolyte leakage data have both shown a positive correlation with MDA production in various studies[186,219,220]. Basil, along with increased electrolyte leakage, when treated with chilling conditions, showed an increase in lipid peroxidation (Figures 3.10 and 3.13) suggesting for its membranes to have experienced damage and therefore plants were sensitive to treatment at 4^0C for 24 h under continuous darkness.

The content of ROS in plant tissues increases under environmental stress conditions that include drought and chilling stress and may be produced to either act as a signalling molecule or as a consequence of reduced enzyme activity[228,252]. Either way, high accumulation of ROS causes damage to cellular components including DNA, lipids, proteins and chlorophyll pigments[105,253]. Hence, it is likely that the increased content of ROS observed in basil in this current study (Figure 3.11 and 3.12) may contribute to the decreased chlorophyll content, increased lipid peroxidation and electrolyte leakage that was observed. Chilling tolerant plants, or plants acclimated to chilling conditions, are known to balance the oxidative stress generated during chilling stress by increasing their

antioxidant defence system. For instance, non-acclimated leaves of winter rye showed inactivation of catalase and PSII under chilling stress (4^0C). However, acclimated leaves showed remarkable photoprotection against oxidative stress by inducing increases in the content of xanthophyll cycle carotenoids, α-tocopherol, ascorbate, glutathione, the activities of superoxide dismutase and glutathione reductase[254]. A chilling sensitive variety of rice, when exposed to chilling temperatures, showed increased electrolyte leakage and MDA production. However, the chilling tolerant variety yielded low electrolyte leakage, MDA production and high antioxidative enzyme antioxidant activity[220]. These findings show the detrimental effect of ROS and their contribution to the development of chilling injury symptoms in plants tissues. The findings also highlight the role of antioxidants in being able to scavenge ROS and provide protection against chilling induced oxidative damage in plants tissues. Basil, however, showed a decline in its antioxidant content after treatment at chilling temperature (Figure 3.14) and that could have allowed the amount of ROS to increase during chilling stress. The reduced antioxidant content during chilling conditions suggests that basil may have lacked the capacity to increase its antioxidant defense system and thereby suffered damaging effects from chilling induced oxidative stress.

Electrolyte leakage results from basil leaves obtained in this current study were in agreement with the findings of other studies that also found increased electrolyte leakage from chilling sensitive varieties of plants (capsicum, coffea, cucumber and tomato) when stored at temperatures less than 10^0C[186,219–222]. Similar to the current study on basil, the increased electrolyte leakage reported in sensitive varieties of coffea, rice and cucumber were also found to have shown increased lipid peroxidation after treatment with chilling temperatures[186,219,220].

The increased accumulation of ROS during chilling stress in chilling sensitive plants was also found to be accompanied with reduced chlorophyll content, lipid peroxidation, reduced water soluble antioxidant content or increased electrolyte leakage[100,219,220]. The results

from physiological assays carried out on basil show the same trends as the findings obtained from other plants that are also considered sensitive to chilling temperatures. Hence, considering the similarities of findings of this current study to those carried out by others on chilling sensitive crops, it appears that basil should be considered to be sensitive to chilling temperatures. The treatment of basil for 24 h at 4^0C is damaging to its cellular components including increased membrane permeability, the decline in protection against oxidative stress and loss of chlorophyll molecules leading for its leaves to show symptoms including strong wilting of the leaves as well as discoloration.

3.3 Conclusion:

The findings of this present study show that basil is sensitive to chilling temperatures as it showed strong wilting of the leaves, loss of membrane integrity, phospholipids degradation, accumulation of ROS, reduced chlorophyll and antioxidants content after storage at chilling temperatures for 24 h. These symptoms are typical of the ones normally observed in a chilling sensitive plant upon storage at chilling temperatures where degradation of cellular components is observed due to them not being able to cope with chilling induced oxidative stress. This present study suggests that transporting basil under continuous dark and chilling conditions for 24 h would normally be detrimental to its appearance and shelf life and thereby affect sales and increase wastage. Treatments that might reduce the chilling sensitivity, thereby allowing basil to be transported at lower temperatures to improve shelf life, would therefore be advantageous to the industry.

4CHANGES OF TRANSCRIPTOMIC PROFILE OF BASIL (*OCIMUM BASILICUM* VAR. *MARIAN*) ON EXPOSURE TO CHILLING TEMPERATURE:

4.1 Introduction:

4.1.1 The importance of understanding molecular changes in plants when stored at low temperatures (chilling or freezing).

The molecular changes in plants during response or acclimation to low temperature stress have been studied intensively using *A. thaliana* and other temperate plants as well as tropical plants[255–257]. Studies investigating molecular changes during freezing stress in *A. thaliana* led to the identification of one of the major cold regulatory pathway – the CBF pathway[64,258,259]. An upregulation of the CBF pathway during cold acclimation in *A. thaliana* had resulted in its increased tolerance to freezing temperatures[64,259]. Hence, the CBF pathway has been used as a target to activate in other plants to increase their tolerance towards low temperatures, either by genetic modification or through applications of light or chemical treatments [68,84,183,260,261].

Knowledge of genetic changes during low temperature stress response in plants is important, as it can used to develop strategies that may be able to improve chilling temperature tolerance in sensitive crops by gene manipulation[262].

4.2 Existence of cold regulatory pathways other than CBF pathway that may contribute to plants tolerance towards low temperature stress.

Mechanisms other than the CBF pathway have been found to have a positive effect on plants' ability to tolerate low temperature. For instance, *A. thaliana* with mutations at the *eskimo1* locus showed an increased freezing tolerance and accumulation of proline without inducing the expression of CBF regulon[263]. This indicates that there may be different signalling pathways in *A. thaliana* that activate different aspects of cold

acclimation to increase freezing tolerance without the activation of other pathways[263]. Thomashow and Fowler[66] investigated transcriptomic changes over hours (1 h, 4 h, 8 h, 12 h 24 h) and 7 days in *A. thaliana* on its transfer from 22°C to 4°C. The study elucidated the existence of mechanisms other than the CBF pathway that were involved in *A.thaliana's* process of cold acclimation. The study showed that there were genes induced at each time point by more than 3 fold that does not fit in with the two step simplistic CBF pathway, where the CBF regulon expression is induced after 4 h of CBF gene activation under chilling temperatures. From the 306 cold responsive genes only 90 genes could be assigned as CBF dependent or CBF independent. From the 90 genes induced, 60 genes were considered CBF independent as they showed an increased expression by at least 3 fold and did not show an increased expression in a CBF overexpressed transgenic *A. thaliana* plant. On the other hand the 30 remaining genes were considered CBF dependent as they showed an increased expression in CBF transgenic and non-transgenic *A. thaliana* plants under chilling temperature. The findings from Thomashow and Fowler[66] suggest the existence of multiple regulatory pathways that are independent of the CBF pathway and also activated during the process of cold acclimation in *A. thaliana*.

4.2.1.1 Transcriptomic changes occurring in metabolic pathways in A. thaliana *on exposure to chilling temperatures:*

Hannah *et al.*[141] looked at changes in metabolic pathways in response to cold temperatures from short (24h), medium (48h) to long term (<120h). Hannah *et al.*[141] found overrepresentation of downregulated genes involved in cell wall modification and lipid metabolism and this was suggested to be a result of reduced growth and development of plants at low temperatures.

Photosynthetic reactions including light reactions and the Calvin cycle were found to be downregulated in the medium and long term[141]. Carbohydrate metabolism

including sucrose synthesis, conversion of UDP-glucose, and starch and sucrose degradation were upregulated in the short term[141]. This may occur to allow for the precursors to enter glycolysis and the TCA cycle that were also found to be upregulated in long term changes observed in *A. thaliana* upon exposure to chilling temperatures. Carbohydrate metabolism was overrepresented among upregulated genes in the medium and long term. These upregulated genes involved in carbohydrate metabolism also include enzymes involved in the production of glucose-6-phosphate, fructose-6-phosphate, glucose, maltose, fructose, sucrose, and trehalose that also accumulated upon exposure to low temperatures[141,264]. Hence, the accumulation of metabolites is also caused by changes in carbohydrate metabolism at times of low temperature stress.

Transport proteins including ATP-binding cassette (ABC) transporters, ATPase, sugar and phosphate transporters located on the plasma, vacuolar and plastid membranes are all upregulated in the short term to increase the redistribution of sugars, PI and ATP around the cell[66,141]. This indicates that the availability of these molecules around the cell may play an important role in plants' initial response to cold temperatures. However, these transportation proteins were downregulated in the long term and this was believed to be due to reprogramming of carbohydrate metabolism causing the availability of sugars in the cell to be re-established over time, rendering their redistribution less important[141].

4.2.1.2 Changes in oxidative status of A. thaliana during storage at chilling temperatures leads to changes in the detoxification system:

Fowler and Thomashow[66] found seven genes responsive to hydrogen peroxide along with enzymes including glutathione-s-transferase and peroxidase to be also upregulated on short term exposure of *A. thaliana* to chilling stress. Secondary

metabolism regulation in the study by Hannah *et al*.[141] also found the overrepresentation of genes coding for flavonoids among upregulated genes across all time points from short to long term on exposure to chilling stress. Flavonoid accumulation in plants at times of chilling stress is known to protect the cells from photo-oxidative stress by absorbing UV light[265]. These changes in antioxidative enzymes and compounds in *A. thaliana* across studies show that the plant experiences increase in its oxidative stress at least in its initial response to chilling temperature stress and that detoxification is induced to combat this stress, indicating that detoxification of ROS plays an important role in cold acclimation.

4.2.1.3 Transcription factor family changes in A. thaliana at times of chilling conditions:

Large global gene expression changes in *A. thaliana* during cold acclimation include several genes coding for transcription factor families[266]. Lee *et al*.[266] and Hannah *et al*.[141] found the AP2/EREBP transcription factor family to be coded by the early cold responsive genes and to be involved in activation of many late cold responsive genes. Affected members of the ARF/DREB transcription family include CBF1, CBF2, CBF3, RAV1 and RAV2 and these are likely to play an important role in plants' response to low temperatures as already mentioned. Other transcription factors induced by late (greater than or equal to 24 h) response to cold include zinc finger, bZIP, Myb, bHLH, HSF and WRKY. bZIP transcription factors play a role in plant pathogen response, light signalling, ABA signalling and abiotic stress signalling. HSF and WRKY transcription factors are normally involved in plants response to heat stress[267] but their upregulation by cold suggests their importance in plants' response to multiple abiotic stress. The upregulation of transcription factor families mentioned above provide us with an insight into their potentially important roles in plants' response to chilling conditions. Such information can prove useful in understanding the mechanisms involved in cold

acclimation and thereby used to improve plants' tolerance towards chilling conditions by gene manipulation.

Considering the useful wealth of information that is obtained from transcriptional profiling of *A. thaliana*, several other transcriptomic profiling studies have also been carried out on other chilling sensitive, economically important crops including rice, *Brassica rapa* and grapefruit to study changes occurring in their response to chilling temperatures[268–270]. These studies found chilling sensitivity to be associated with repression of genes involved in various cellular activities and for chilling adaptation to involve upregulation of genes involved in metabolic pathways[268,270]. Such information provided valuable knowledge on candidate genes that can be used to improve crops' tolerance towards chilling temperatures through genetic engineering or biotechnology. Similarly, other crops such as tomato and *Brassica napus* have also benefited from studies carried out on *A. thaliana* that elucidated the existence of the CBF pathway to increase plants' tolerance towards low temperatures. When *A. thaliana*'s CBF genes were overexpressed in Tomato and *Brassica napus* these plants were found to show significantly greater chilling tolerance than wild type crops[82,271]. This shows that the trait of increased tolerance towards chilling temperatures is transferrable in otherwise sensitive plants through biotechnology. However, in this present study, the aim was not to genetically modify the basil plants to improve its tolerance towards chilling temperatures but to simply understand the changes occurring at molecular level in response to chilling conditions. The analysis of transcriptomic changes could then be used to identify ways that may be suitable in improving basil's tolerance towards chilling temperatures.

In order to establish ways to improve tolerance of basil towards chilling temperatures it was also important to understand the changes occurring in basil at a genetic level on its exposure to chilling stress. Hence, this chapter aims to establish the transcriptomic

changes occurring in basil on exposure to 24 h of chilling stress by carrying out RNA-sequencing analysis on basil's total RNA exposed to either 24 h of ambient or 24 h of cold at 12 h photoperiod.

In the course of writing this chapter, Zhan *et al.*[272] recently published the findings of transcriptomic changes in basil (*Ocimum americanum var. pilosum*) in response to chilling conditions. Zhan *et al.* (2016) have identified overrepresented biological processes, cellular components, molecular functions and Kegg pathways among differentially-expressed genes compared with a background population. The study by Zhan *et al.*[272] bears similarity with the findings of this study. In addition, while the study by Zhan *et al.*[272] identifies gene ontology parent terms, this study describes more specific and detailed overrepresented child terms among differentially-expressed genes compared to the background population in response to chilling conditions. This study, however, also differs from that of Zhan *et al.*[272] in one of their major findings regarding the CBF pathway. The CBF pathway was not found in basil's sequencing data in this study, possibly due to the stringent transcript identification criteria used in this study while Zhan *et al.*[272] not only identified CBF transcription factors but claimed basil's inability to induce CBF's expression in response to chilling conditions to be the factor accountable in basil's sensitivity towards chilling conditions.

This present study identifies potential pathways, biological processes, cellular components and genes with roles of importance in basil's transcriptomic response to chilling temperatures. It also identifies the possible mechanisms employed by basil that are involved in either protection or susceptibility towards chilling conditions. However, as is the case for many RNA-seq based transcriptomic studies that have been carried out without replication, this study has also been conducted with the use of only single replicate of each sample. In this present study, similar to other studies, RNA-seq was used for pre-screening basil's transcriptomic profile in response to chilling

conditions to identify candidate genes to be investigated further by way of qRT-PCR. In addition, the transcriptome of basil has not yet been sequenced, annotated and is not part of any database. Most significantly, there is no database of gene ontology for basil. Therefore, the gene ontology enrichment analysis was carried out on basil's differentially expressed genes based on homology to genes in *A. thaliana's* annotated transcriptome as its background. Therefore, findings from this study simply guides the reader with transcriptomic changes possibly relevant in basil's response to chilling conditions and can provide valuable biological information. However, any information extracted from this study should be investigated further by qPCR and other supporting physiological experiments before making any conclusions. Nonetheless, qPCR analysis for a small sample of representative genes concurred with the transcriptomic analysis, providing a degree of validation of the RNA seq data.

In this study the aim is to analyse transcriptomic changes in terms of biological processes, metabolic pathways, cellular components affected and mechanisms involved in coping or exhibiting susceptibility towards chilling stress.

4.3 Results and Discussion

To investigate the transcriptomic changes occurring in basil after storage at chilling temperatures for 24 h, approximately 32 days old basil plants were brought from growers site (Vitacress) during summer season and transferred into their ambient (22^0C) or chilling conditions (4^0C) at 12 h photoperiod. Basil plants were transferred at ZT23 and harvested at ZT23 (Figure 4.1).

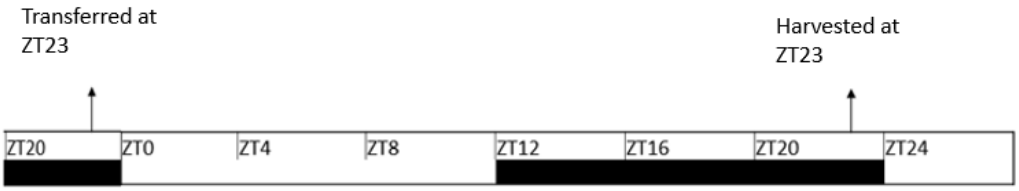


Figure 4.1 Shows transfer and harvest time points of basil plants stored under ambient or chilling conditions for 24 h at 12 h photoperiod.

4.3.1 Illumina sequencing raw data

Two RNA samples of basil stored under ambient or chilling conditions were sequenced using pair end high throughput illumina Hiseq2500 sequencing technique in Cleveland clinic, Ohio and its raw reads were quality checked using fastqc software.

Approximately 8 million reads were produced from the basil sample stored at ambient conditions and 9 million reads were obtained from the chilling treated sample. A FastQC quality check of these samples showed that the length of each read was 44 bases long after removal of indexing primers. The per base sequence quality check was analysed using the Illumina v 1.9 encoding method. It was found that in the reads of 44 bases the individual bases within the read had been called with average Phred quality score (Q score)[1] of at least 37. This means that there is 99.95% of probability that the bases of each read were called accurately. The average score per sequence of the library sequences was calculated by using scores of each base within a sequence and calculating their mean per sequence that was also 37 (Figure 4.2). These means per sequences were used to plot the distribution of their quality scores. It was found that all of the sequences in the library were called accurately by the sequencer as the

[1] Phred quality score is a parameter used by illumina to determine the accuracy of nucleotide or base call by the sequencer and defined as a property that is logarithmically related to the base calling error probabilities (P)2. Q score can be calculated using the formula $Q = -10 \log_{10} P$. A score of 10 represents that there is a probability of 1 base being called incorrectly in every 10 bases and a score of 20 will indicate a probability of every 100 base being called incorrectly in a read. Hence, a threshold of 30 quality score is used to consider a base call accurate (99.9) [448].

distribution of all of the sequences formed a very tight single distribution at a high score of 39 (Figure 4.2). An absence of bump in the graph indicated that there was not any population of sequences in the library that was sequenced poorly or needed to be trimmed.

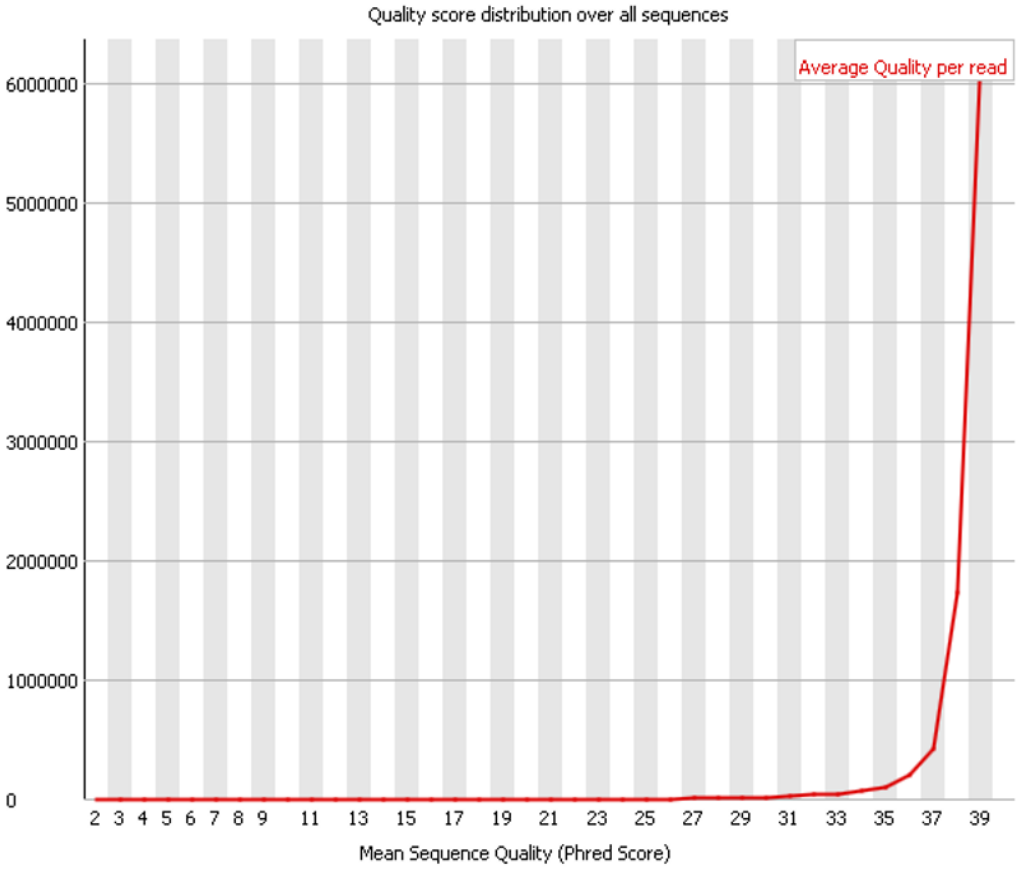


Figure 4.2 Quality control check analysis using FastQC software showed that the average quality of base call across reads are of score 37.

4.3.2 Alignment and assembly

Alignment and assembly of the raw sequence data into transcripts was performed by Dr Sandra Smieszek (Case Western Reserve University, Ohio). Genome-guided de-novo assembly was performed using the Trinity software[273] in order to assemble basil's reads into transcripts. This involved first mapping the raw reads to *A. thaliana's* transcriptome so that they are grouped according to locus. This was then followed by de novo transcriptome assembly at each locus. Here, the genome is only used as a

substrate for grouping overlapping reads into clusters then the basil transcripts are reconstructed based on the actual read sequences but can be assigned to (annotated as) *A. thaliana* orthologues. The Trinity software aligned by 50% of the basil reads to *A. thaliana* and the remaining reads that did not map were discarded. The resulting transcripts were annotated to *A. thaliana's* genes. The use of *A. thaliana* plant as a reference transcriptome was due to the fact that it is a model plant to many researchers, intensively studied and thus a better annotated organism than other plants Hence, the use of *A. thaliana* as a reference genome was believed to have allowed us to analyse transcriptomic changes occurring in basil in greater detail and in various annotation categories, particularly gene ontology, that could otherwise be limited if the transcripts were annotated to other less comprehensively annotated crops. An alternative possibility might have been to align to tomato. Tomato shares a much greater similarity with basil than *A. thaliana* does meaning that more than 50% of the reads may have been aligned[274]. This is clearly demonstrated via a phylogenetic tree constructed using the *FAD6* gene sequence which was among those genes identified in the basil RNA seq data. *FAD6* is a sequence which is available for a large number of plant species. The phylogenetic tree constructed using *FAD6* gene showed that basil was most closely related to tomato and potato (Figure 4.3). In contrast, basil's relation with *A. thaliana* was more distant than it was with tomato, as a result, a lot of basil's transcripts may have possibly been discarded that if aligned to tomato may have been picked up. However, the tomato genes to which the reads would have been assigned have little available ontology information making further analysis difficult. In addition, the first RNA sequencing experiment and analysis were carried out in 2013 when RNA sequencing was first introduced. The annotations of tomato's genes at the time was spread out across different databases and not integrated into a single window. Thus, annotation of basil's transcripts to tomatoes genes would make the interpretation of biological changes difficult, less comprehensive and less accurate

due to the possible heterogeneity of annotations across different databases. Another possibility might be to use entirely de novo assembly which would not discard any sequences. This could be followed by a subsequent large scale BLAST search to assign all transcripts to an *A. thaliana* orthologue. Running a separate BLAST alignment also has the advantage of allowing the use of protein sequence for BLAST alignment. After assembly, basil RNA sequences can be translated before BLAST into protein sequences which are generally better conserved between species than RNA sequences, possibly identifying additional *A. thaliana* orthologues which would not be similar enough to meet the cut-off at the RNA level. This entirely de novo approach would have required considerable extra computing power. However, more recent advancements in the field of next generation sequencing analysis have since made this option more available and this meant it was possible to do this for subsequent RNA sequencing experiments (see chapter 6).

There were 7978 sequences successfully aligned with a subject sequence in the database. After these sequences were filtered to remove any that had a bit score of ≤ 80, the new filtered list contained 4633 sequences. After duplicate AGI ID's were averaged based on their expression values, the list of 4633 transcripts was further shortened to the current list containing 3000 transcripts only. The transcriptomic profile of basil mapped to *A. thaliana* transcriptome was found to have generated 3000 transcripts with significantly high homology with that of *A. thaliana* transcripts. From the sequenced 3000 individual transcripts, 1198 (40%), of the entire transcriptome showed at least a two-fold change in expression in response to chilling temperature. Of these chilling responsive genes, 137 (11%) of the genes were upregulated and 1061 (89%) were downregulated.

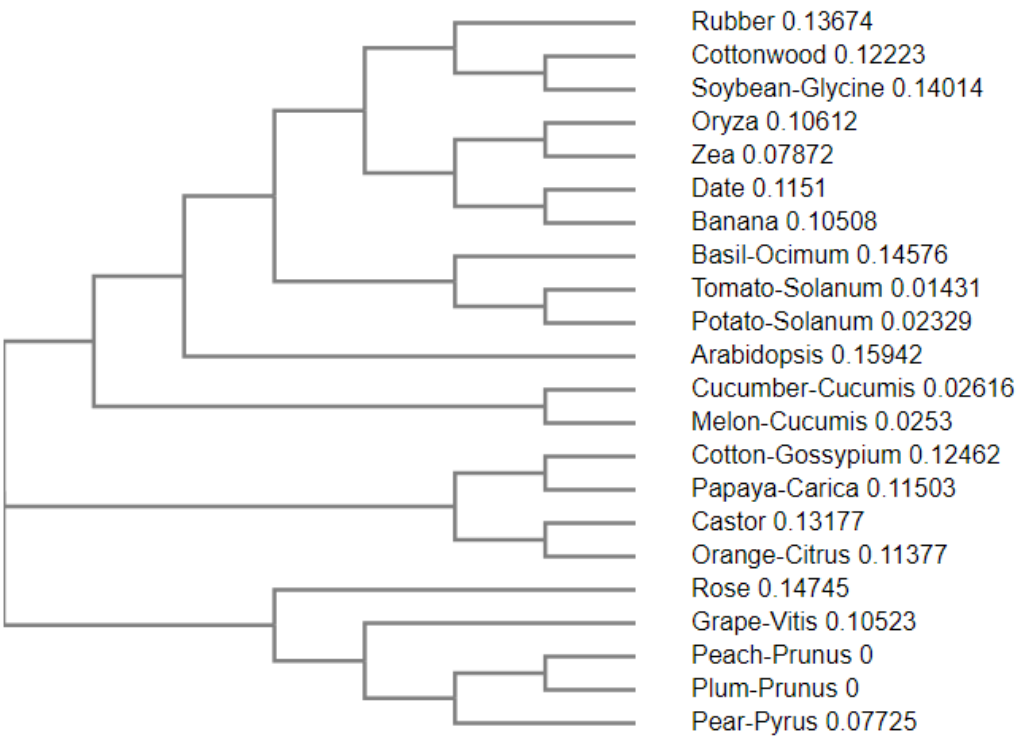
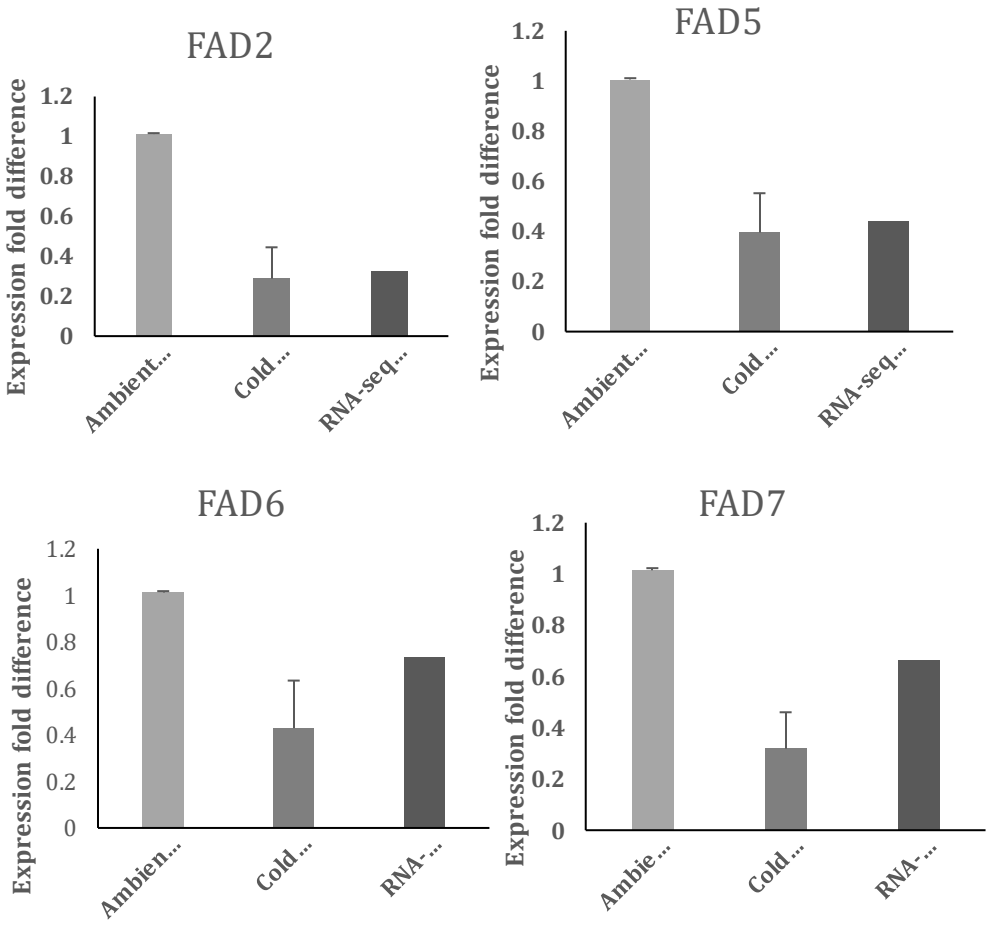


Figure 4.3 Shows phylogenetic tree of 22 organisms constructed using FAD6 (1579 bp in basil) mRNA sequence that shows basil's relationship with other organisms.

4.3.3 Determination of reproducibility of RNA sequencing expression data via qRT-PCR.

The biological information interpreted from the RNA seq data were based on single replicates of basil samples. Hence, a series of qPCR gene expression analyses were carried out to identify the reproducibility and thereby genuinity of RNA seq data. Gene members of the fatty acid desaturase (FADS) family, were used for measurement of differential gene expression by qPCR to determine the reproducibility of results obtained from the RNA sequencing data. Five genes including FAD2, FAD5, FAD6, FAD7 and FAD8 were analysed, among which FAD2 and FAD5 genes showed downregulated expression in RNA seq data by 0.322 and 0.44, respectively (Figure 4.4). FAD6, FAD7 and FAD8 genes, on the other hand, consisted of low gene expression fold difference by 0.73, 0.663 and 0.749, respectively, in response to chilling conditions in the RNA seq data (Figure 4.4). The results obtained from qPCR revealed that FAD2, FAD5, FAD6,

FAD7 and FAD8, were downregulated in their gene expression by 0.288, 0.395, 0.428, 0.318 and 0.194, respectively, (Figure 4.4). The downregulation of FAD5, FAD6, FAD7 and FAD8 genes occurred to a greater extent when measured by qPCR than observed in RNA seq data. The difference in the magnitude of downregulation seen in qPCR and RNA seq data could perhaps be due to qPCR being a more sensitive technique for measurement of differential gene expression data than RNA seq technique. The similarity observed from FADS differential gene expression measurements across RNA seq, and qPCR technique suggests that the data recorded by RNA seq is likely to be reliable and reproducible.



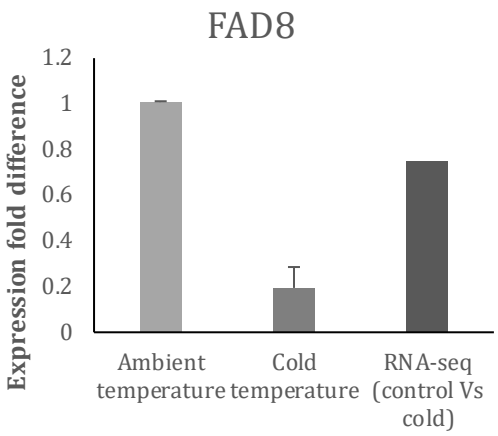


Figure 4.4 Differential gene expression in fold difference of fatty acid desaturases determined by qRT-PCR. Each bar represents mean of n = 3 and the error bars show standard error. RNAseq data is added for comparison.

4.3.4 Gene functional classification:

The gene classification tool of DAVID was used to extract the biological information behind the differentially expressed list of genes. The tool allows the researcher to condense the quantitative data into a few clusters based on gene ontology and view at a glance the biological information captured by the high-throughput technology. The genes are classified into clusters using the novel Heuristic Multiple Linkage algorithms that assess the functional similarity between pairs of genes and groups them into a cluster based on that function or group of funcrions. The significance of the relationship between the genes is estimated using a kappa statistical method which measures the degree of agreement between a range of different information sources. The use of 14 functional annotation sources to collect this information makes DAVID superior to other classification software. A score of 0.35 or above indicates a significant similarity and a value close or equal to 1 represents close relationship between gene members. Only genes that are closely related to each other become part of a cluster, and the same gene can participate in more than one cluster. The researcher can adjust the threshold of Kappa and make the criteria for gene inclusion in a cluster more stringent. However, in this present chapter, a default of 0.35 Kappa threshold was used, as a higher value could increase the number of clusters, reduce

the condensation of genes and make the biological information more spread out. Clusters can then be analysed to determine whether there is an enrichment in terms of the proportion of differentially expressed genes in a cluster compared to the proportion of genes found in that cluster for the whole genome.

4.3.5 Gene ontology classification overview of differentially-expressed genes (both up and downregulated)

The 1198 cold responsive genes (up and down-regulated) were classified into 34 different clusters based on gene functional classification at the level of overall biological terms with significant enrichment score (≥ 1 fold enrichment and p value <0.05[2]). The top 15 clusters with high enrichment score are shown in table 4.1 that includes gene groups associated with functions for chloroplast components: chloroplast envelope, chloroplast thylakoid, thylakoid membrane and envelope of both chloroplast and plastid (Table 4.1). They also include the terms: membrane in general, organelle membrane, organelle envelope, intrinsic and integral to membrane. The genes were also involved in processes including chlorophyll biosynthetic and metabolic process, cellular homeostasis, oxidation-reduction and fatty acid biosynthetic process (Table 4.1). The overrepresented activities of genes have been clustered into those that include transportation of ions, cations, isomerase and transmembrane transport activity (Table 4.1). This finding suggests that chloroplast and its components along with fatty acid, amino acid synthesis and transportation of ions to be relevant in basil's cellular response to chilling temperatures.

[2] A modified Fisher Exact P value was used to assess the significance of enrichment of genes association with annotation terms[449]. The Fisher's Exact test is believed to be better in assessing significance of gene enrichment with annotation terms from a small list of genes[207]. Hence, DAVID calculates a modified Fisher's Exact p value by EASE (Expression Analysis Systematic Explorer) score that improves the accuracy of enrichment P value calculations[450]. A P value of 0 represents significant enrichment where as a value of 0.05 or above indicates enrichment of genes with the annotation terms to be no more than by random chance[449].

Gene fucntional classification: Biological terms	Enrichment score	Genes
Chloroplast envolope, organelle envolope, chloroplast, chloroplast part or plastid part	18.74	AT2G34460;AT2G32040;AT1G76405;AT1G50900;AT5G26820; AT5G35970;AT5G64940;AT1G15500;AT2G35800;AT1G08570; AT3G26570;AT2G45990;AT1G30360;AT1G67700;AT2G38040; AT3G25690;AT2G45290;AT3G06510;AT2G43950;AT4G30620; AT5G39410;AT3G12570;AT5G22830;AT5G64290;AT1G70410; AT1G17160;AT1G16720;AT2G34590;AT3G09580;AT1G78620; AT3G55030;AT5G66530;AT2G33180;AT1G48350;AT4G31780; AT1G13930;AT3G17040;AT3G48850;AT5G33320
Thylakoid memebrane, chloroplast thyakoid, organelle membrane	12.77	AT2G34460;AT2G39730;AT1G76405;AT3G29240;AT2G17972; AT1G50900;AT2G37220;AT3G56140;AT5G13120;AT5G64940; AT5G37360;AT1G22700;AT3G56940;AT1G20340;AT5G20720; AT2G45990;AT1G67700;AT5G64040;AT1G15820;AT5G36120; AT1G03600;AT2G45290;AT2G10940;AT3G27830;AT3G45140; AT1G17160;AT3G57680;AT4G37200;AT1G14345;AT5G01600; AT1G08550;AT2G47450;AT1G68830;AT1G74470;AT1G34000; AT3G09050;AT3G26580;AT5G02160;AT5G66530;AT5G42070; AT2G33180;AT3G63490;AT5G12130;
Co-factor biosynthetic process, porphyrin biosynthetic and metabolic process, chlorophyll biosynthetic and metabolic process.	11.52	AT2G34460;AT4G01690;AT3G48730;AT1G74470;AT1G58290; AT3G59400;AT4G15560;AT1G44446;AT1G09940;AT3G14930; AT1G63970;AT2G40490;AT3G56940;AT5G08280
Protein folding and heat shock proteins	9.29	AT1G28150;AT2G30100;AT4G09350;AT4G13830;AT1G80920; AT3G47650;AT4G13830;AT2G30100;AT1G77930
Transmembrane region, integral or intrinsic membrane, transmembrane transporter activity	8.92	AT1G23090;AT3G06450;AT1G23090;AT5G13550;AT1G15500; AT3G26570;AT5G54800;AT1G77990;AT1G15500;AT1G77990; AT5G20380;AT5G13550;AT3G26570;
Oxidation-reduction, redox active centre, cellular homeostasis, thioredoxin and electron trasnport chain	8.6	AT2G39730;AT3G11630;AT3G51030;AT5G06290;AT2G38270; AT1G65980;AT5G20720;AT3G02730;AT1G08570;AT4G04950
Amino acid biosynthetic process & organic acid biosynthetic process	8.28	AT3G61440;AT1G48850;AT1G10070;AT2G14750;AT1G72810; AT4G29840;AT3G60880;AT3G01120
Membrane, mitochondrial membrane, mitochondrial envolope and mitochondrial substrate carrier.	7.04	AT3G05290;AT5G14040;AT1G72820;AT5G39410;AT2G35800; AT1G15500;AT1G74240;AT3G48850;AT5G33320;AT4G01100; AT5G17400;AT5G46800;AT5G08740
Pigment metabolic process, pigment biosynthetic process, biosynthesis of plant hormones, terpenoid and isoprenoid biosynthetic and metaboic process	6.72	AT4G14210;AT1G63970;AT3G10230;AT5G67030;AT4G25700; AT5G61670;AT1G06820
Plastid envolope, chloroplast, outer membrane, organelle envolope, plastid part, plastid outermembrane, intracellular transport, intrinsic and ntgral to membrane	6.7	AT4G35450;AT3G06510;AT2G35800;AT3G46740;AT2G28900
Cytoplasmic, cell membrane, plasma membrane, itrinisic and integral too membrane or involved in trasnportation.	5.65	AT2G36830;AT4G10380;AT4G16480;AT1G11260;AT5G63850; AT1G77210;AT1G01620;AT3G53420;AT3G43790;AT2G48020; AT2G26510;AT4G35100
Isomerase activity, plasma membrane & cytosol	4.95	AT4G34960;AT2G16600;AT4G38740;AT1G74070
Transmembrane region, integral or intrinsic membrane, sugar and auxin transport protein	4.81	AT2G40420;AT5G53760;AT1G77690;AT5G63850;AT1G31830; AT1G12640;AT2G35760;AT1G63260;AT1G17200;AT2G24220; AT1G11260;AT3G56200;AT1G63010;AT1G54320;AT4G39220; AT1G59740;AT3G12360;AT2G38120;AT2G37330;AT1G52870; AT5G13760;AT5G01240;AT3G48850;AT2G26510;AT5G20380
Fatty acid biosynthetic process	4.21	AT4G00400;AT3G12120;AT2G38110;AT2G26250;AT1G68530; AT1G01610;AT1G19440
Transport, cation transport, metal ion transport and transmembrane transport activity.	4.19	AT5G11800;AT3G06450;AT2G46800;AT1G78920;AT3G58810; AT1G16780;AT1G70300;AT3G02050;AT5G14880;AT4G30560; AT1G60960;AT2G35060;AT4G13510;AT4G22200

Table 4.1 Enriched Gene ontology terms associated with differentially regulated genes (up and down-regulated) in basil's response to chilling conditions based on DAVID analysis (p value <0.05 based on Fisher's Exact p value by EASE). AGI codes of *A. thaliana* genes to which basil genes had been mapped are listed.

4.3.6 Detailed analyses of biological process, cellular component and molecular function classification of up and downregulated genes

4.3.6.1 Biological processes that have been identified as overrepresented among up-regulated genes:

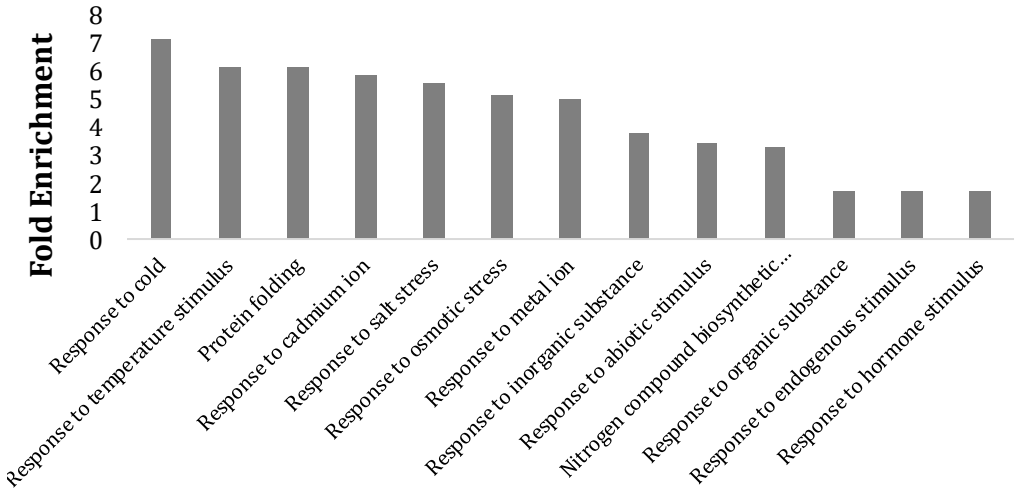


Figure 4.5 DAVID functional annotation of the biological processes found to be significantly (P value < 0.05 based on Fisher's Exact p value by EASE) overrepresented among basil cold-upregulated genes, compared to the gene population background.

To gain a clear understanding of the roles that these differentially regulated genesmay play in response to chilling temperatures, further, separate gene ontology analyses was carried out for each of the categories, biological processes, cellular component and molecular function, in even more detail, examining up and downregulated genes separately. Each gene ontology term found in each category of gene ontology was selected as enriched[3] when it reached a fold enrichment score of ≥1 and count of genes being at least 10 (Figure 4.5).

[3] Enrichment is a term used to describe overrepresentation of genes in the user's list compared to the background population. For instance, 10% of the user's genes are kinases when in a human genome there are only 1% of kinases. Hence, the user's gene list is considered to be enriched with kinases where the significance of the overrepresentation is determined by the use of statistical method such as Chi-squared test or Benjamin test[207].

For the cold upregulated genes, 13 gene ontology terms belonging to the biological process categories, response to cold, response to temperature stimulus, protein folding, response to cadmium ion, response to salt stress, response to osmotic stress, response to metal ion, response to inorganic substances, response to abiotic stimulus, nitrogen compound biosynthetic process, response to organic substance, response to endogenous stimulus and response to hormone stimulus categories were found to be significantly overrepresented (Figure 4.5) (details of genes annotated to each GO term are provided in appendix 4.1). The enriched biological processes described in Marion basil are mostly child terms of the parent term "response to stimulus" that was also reported by Zhan *et al.*[272] as a biological process GO enriched term in *O. americanum* var. pilosum basil under chilling conditions. Among the enriched biological processes of Marion basil, the GO terms that have been commonly reported as playing a role in the plant's response to chilling temperatures are response to cold, temperature stress, osmotic stress and salt stress[275]. The list of biological processes enriched in basil's response to chilling temperatures also includes other undocumented biological processes such as response to cadmium ion, metal ion and response to inorganic substance that will be discussed later in this chapter.

Many genes annotated as being involved in cold response are also annotated to be involved in temperature stress and abiotic stress. Hence, the functional annotation clustering tool in DAVID was used to identify the annotation terms that share relationship with one another and thereby group the various biological processes into clusters. The relationship between annotation terms was measured by the extent of their co-association with genes. The functional annotation clustering of upregulated genes identified three most enriched clusters that includes cluster 1 (response to cold, response to temperature stimulus and response to abiotic stimulus; enrichment score

7.78), cluster 2 (response to abiotic stimulus, response to salt stress and response to osmotic stress; enrichment score 7.35) and cluster 3 (response to cadmium ion, response to metal ion and response to inorganic substance; enrichment score 5.71).

Based on the *A. thaliana* mapping, basil genes that were annotated to be associated with the biological processes of cluster 1 are arginine decarboxylase 1, arginine decarboxylase, 2-cys peroxiredoxin BAS1, a WRKY transcription factor 33, a heat shock protein 70, a 20KDa protein, alpha glucan water dikinase 1 and glycine-rich RNA binding protein 7 (GRP7 also called cold clock regulated 2). The above mentioned eight genes have been suggested to play a role in plants tolerance to different types environmental stresses such as salt stress, wound, peroxide detoxification, drought, heat, and cold stress, respectively[276–282].

The eight genes, arginine decarboxylase 1, arginine decarboxylase 2, 2 cys perrodoxin BAS1, the WRKY transcription factor 33, the heat shock protein 70, the 20KDa protein, alpha glucan water dikinase 1 and GRP7 code for proteins with a range of different properties that can increase a cell's resistance to damage by chilling temperature. For instance, arginine decarboxylase 1 and arginine decarboxylase 2 are involved in the synthesis of polyamines[277] that along with 2 cys peroxiredoxin possess antioxidative properties and are able to reduce cellular ROS toxicity[283,284]. In addition, WRKY transcription factor 33 causes induction of stress responsive genes while heat shock protein 70 and 20 KDa chaperone proteins assist and prevent other proteins from misfolding during temperature stress[278–280]. Alpha glucan water dikinase phosphorylates starch and increases its accessibility for degradation by starch degrading enzymes. Mutants of alpha glucan water dikinase showed lack of accumulation of soluble sugars including glucose and fructose and impaired freeing tolerance in *A. thaliana*[285]. However, the effects were reversed and freezing tolerance was improved when alpha glucan water dikinase was overexpressed. Alpha glucan

water dikinase has therefore been suggested to regulate accumulation of glucose in cells and thereby provide cells with greater stability against damage by chilling temperature[285,286]. Lastly, GRP7 prevents water loss and alleviates dehydration during salt and cold stress by controlling the opening and closing of the stomata in the guard cells[282].

Cluster 1 is the most enriched among other clusters and therefore identifies genes of potential highest importance with roles in chilling tolerance and also suggests that basil is likely to have experienced oxidative stress as there was activation of genes involved in ROS's detoxification.

In Cluster 2 (response to abiotic stimulus, response to salt stress and response to osmotic stress, enrichment score 7.35) the co-associated genes among these responses were aconitate hydratase 1, aconitate hydratase 2, WRKY transcription factor 33, an ATP synthase, heat shock protein 81-2, a fructose bisphosphate aldolase, arginine decarboxylase 2 and a glycine rich RNA binding protein. Among these genes aconitate hydratase 1, aconitate hydratase 2 and fructose-2, 6-bisphosphatase are involved in TCA cycle and glycolysis, but are also involved in the antioxidant defence system. Cluster 3 (response to cadmium ion, response to metal ion and response to inorganic substance) co-regulated genes include aconitate hydratase 2, a Heat shock cognate 70 kDa protein, phospholipase D beta , peroxiredoxin-2B , a Fructose-bisphosphate aldolase, a Peptidyl-prolyl cis-trans isomerase CYP, a 20 kDa chaperonin, a Formate dehydrogenase and glycine-rich RNA-binding protein 7. Among these genes phospholipase D β, peroxiredoxin -2B, the peptidyl-prolyl cis-trans isomerase CYP and the formate dehydrogenase are involved in hydrolysis of phospholipids, reduction of hydrogen peroxide, folding of proteins and reduction of NAD+ to NADH[287–290]. These genes code for proteins that play protective roles in plants response to stress conditions such as oxidative stress and metal ion stress by limiting the effects of ROS.

Thus, the increased expression of β, peroxiredoxin -2B, the peptidyl-prolyl cis-trans isomerase CYP and the formate dehydrogenase genes can also be possibly beneficial for protection of the basil plants under chilling stress which are also likely to undergo oxidative stress.

Salt stress and metal ion stress causes increased oxidative stress and inhibition of enzyme activity involved in signalling, ion transport, energy metabolism leading to reduced photosynthesis and growth[291]. These are similar damaging effects to those experienced by chilling sensitive plants at chilling temperatures[87]. Hence, induction of genes involved in promoting tolerance of plants towards these stresses could also provide protection against injuries induced by chilling stress. From 19 commonly regulated genes identified, at least 8 showed some role in redox signalling and in limiting the oxidative stress generated in the cell. This shows that basil in response to chilling temperature was likely to have suffered oxidative stress and to have caused activation of various genes involved in reducing accumulation of ROS. Hence, genes commonly regulated within these 3 enriched clusters and particularly those (Arginine decarboxylase 1, arginine decarboxylase 2, heat shock cognate 70 kDa protein, heat shock protein 81-2, 2-Cys peroxiredoxin BAS1, aconitate hydratase, fructose-bisphosphate aldolase and peroxiredoxin) possessing antioxidative properties are of potential importance to be investigated further for their beneficial role in promoting chilling tolerance in chilling sensitive basil.

4.3.6.2 Biological processes identified as overrepresented among downregulated genes:

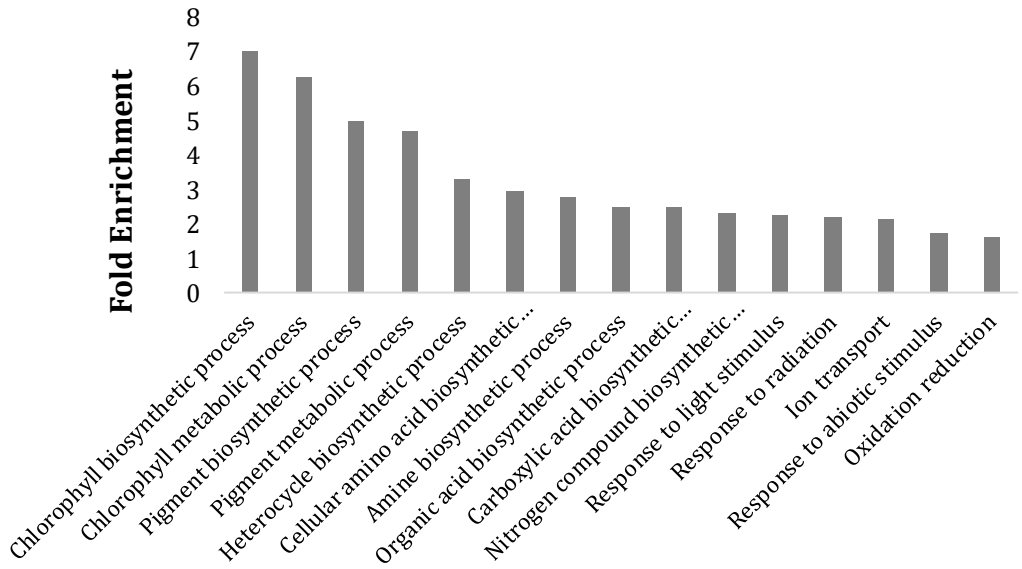


Figure 4.6 DAVID functional annotation of genes found to be significantly (P value < 0.05 based on Fisher's Exact p value by EASE) overrepresented among basil cold downregulated genes, compared to the gene population background.

38 different biological processes where found as significantly enriched GO terms compared to the background population in response to chilling conditions among downregulated genes. However, only the top most significant 15 enriched GO terms among downregulated genes in response to cold are discussed. These include chlorophyll biosynthetic process, chlorophyll metabolic process, pigment biosynthetic process, pigment metabolic process, heterocycle biosynthetic process, cellular amino acid biosynthetic process, amine biosynthetic process, organic acid biosynthetic process, carboxylic acid biosynthetic process, nitrogen compound biosynthetic process, response to light stimulus, response to radiation, ion transport, response to abiotic stimulus and oxidation reduction (Figure 4.6) (details of genes annotated to each GO term and description of remaining 23 GO terms are provided in appendix 4.2). The gene ontology terms from the category of biological processes were analysed

using the tool DAVID functional annotation clustering that yielded three enriched clusters with enrichment scores of 6.3, 4.63 and 3.9.

Cluster 1 consisted of response to light, abiotic stimulus and responses to radiation biological processes. The genes annotated to biological processes of cluster 1 include proton gradient regulation (PGR5), glutamate receptor 3.6, SPA1, COP1 and catalase 1 and catalase 2. PGR5, glutamate receptor, catalase 1 and catalase 2 in *A. thaliana* have been indicated to play roles in the prevention of the cells from damage by environmental stress. PGR5 is involved in photoprotection of PSI by regulating the flow of electrons from ferredoxin to plastoquinone in cyclic electron transportation[292] while catalase 1 and catalase 2 are involved in detoxification of ROS[293]. Glutamate receptor 3.6 has been proposed to be involved in light-signal transduction[294] and calcium homeostasis via the regulation of calcium influx into cells[295]. Downregulation of glutamate receptor 3.6 may possibly contribute to basil's susceptibility to chilling temperatures as an influx of Ca^{2+} ions and light are both required for the expression of several cold-regulated genes (CORs)[296]. SPA1 and COP1 are suppressors of plant photomorphogenesis, and their downregulation may increase basil's cells' adaptation to chilling temperature. A reduced COP1 activity can cause accumulation of SIZ1 genes. SIZ1 is an E3 SUMO ligase that conjugate SUMO functional groups onto the residues of the target proteins thereby stabilises them and influences their function[297]. Overexpression of SIZ1 has led to increased tolerance in *A. thaliana* plants to cold, salt heat and dehydration stress while knockout mutant of SIZ1 resulted in increased susceptibility to low temperature, salt and heat stress[297]. Hence, it may be possible that downregulation of COP1 in basil cells post chilling stress may enhance its capacity to adapt to chilling temperatures while decreased expression of PGR5, glutamate receptor 3.6, catalase 1 and catalase 2 may increase basil's cells' susceptibility to chilling stress and, if so, could be an unwanted response.

Cluster 2 consists of the biological processes pigment metabolic process, pigment biosynthetic process, chlorophyll biosynthetic, chlorophyll metabolic process, heterocycle biosynthetic process, cofactor metabolic process, porphyrin metabolic process, tetrapyrrole metabolic process & porphyrin biosynthesis. Genes that were similarly annotated to cluster 2 include those that are involved in chlorophyll and phytochromobilin synthesis (protoporphyrinogen oxidase, glutamyl - tRNA reductase 1 and glutamyl – tRNA reductase 2)[298–300].

In a study by Aghaee *et al*[301], a chilling sensitive genotype of rice showed a decrease in total chlorophyll content while chilling tolerant genotype did not and was believed to have occurred as a result of oxidative stress generated during chilling stress in sensitive plants. An association between chilling tolerance and chlorophyll accumulation was also suggested[301], however, the mechanism through which chlorophyll accumulation may contribute to an increased cold tolerance in plants is not yet known. Hence, downregulation of genes involved in chlorophyll synthesis in basil cells indicates a trait of a chilling sensitive plant and provides support on the suggestion of chlorophyll accumulation to be possibly associated with cold acclimation.

Cluster 3 (organic acid biosynthetic process, nitrogen compound biosynthetic process, cellular amino acid biosynthetic process, amine biosynthetic process; enrichment score 3.91) contains similarly annotated to genes such as homocystine-s-methyltransferase 3, homocyetseine-s-methyltrasnferase 1 and homoserine dehydrogenase. However, limited literature is available in understanding their impact on plant response to chilling temperatures, hence, these genes will not be discussed further.

Furthermore, 89 genes were annotated to be associated to the term oxidation reduction that was also found as enriched among downregulated genes. These genes coded redox proteins such as ferredoxin and thioredoxin and antioxidative enzymes

including nitrate reductase, peroxidase and glutathione peroxidase. This shows that genes coding for proteins involved in detoxification of ROS were supressed in their expression and provide a possible explanation to the increased ROS accumulation in basil cells post chilling stress shown in the previous chapter. The gene ontology of downregulated genes has identified possible mechanisms that may account for Marian basil's inability to tolerate low temperatures including downregulation of genes involved in mediating light responses and reduced ability to response to oxidative stress.

4.3.6.3 Cellular components overrepresented among differentially expressed genes

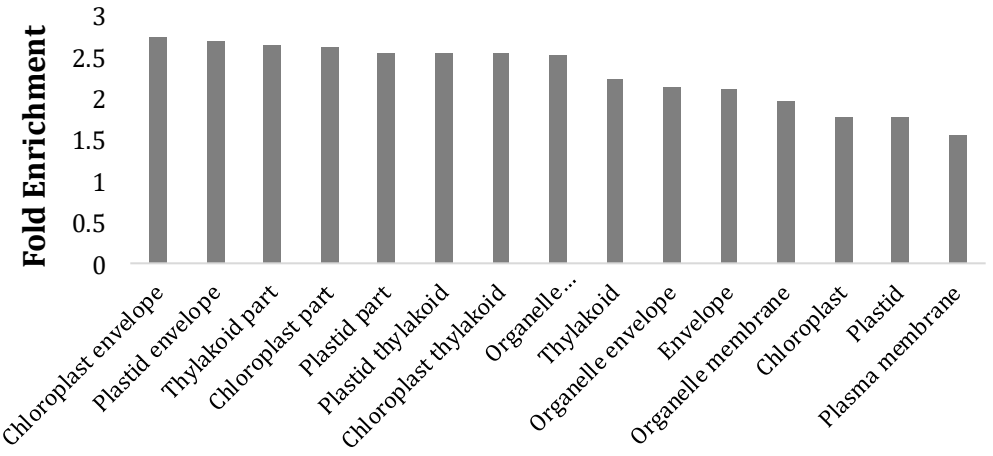


Figure 4.7 DAVID cellular component annotation of genes shown to be significantly (P value < 0.05) overrepresented among basil cold differentially regulated genes.

The enrichment analysis of up and downregulated genes identified many of them to be associated to chloroplast. Hence, the enrichment of genes associated with the cell and cellular components was investigated in more detail among up and down-regulated genes. Chloroplast envelope, chloroplast thylakoid, chloroplast part, plastid part, plastid thylakoid, organelle envelope, organelle membrane and plasma membrane (Figure 4.7 and 4.8) were identified as the 15 of the 30 cellular components that were overrepresented as being associated with differentially expressed genes (Up

and down-regulated) (Figure 4.7) (details of genes annotated to each of GO term and description of all 30 overrepresented GO terms are provided in appendix 4.3). According to the count of genes, the cellular components to which the genes were most associated to include plastid, chloroplast and plasma membrane indicating for these components to have been most affected by drop in the temperature of the environment (Figure 4.7). For each cellular component there was greater downregulation of genes than upregulation. Majority of the genes annotated to chloroplast were involved in either chlorophyll or carbohydrate metabolism or in detoxification of free radicals while genes annotated to plasma membrane coded for proteins involved in mediating signalling pathways, transportation of ions, water molecules, sugars and vesicles.

Chloroplast and plasma membrane in chilling intolerant plants are the two most sensitive sites towards low temperature (Figure 4.7)[242,302]. In this study 295 (25%) of the differentially expressed genes were annotated to chloroplast and 184 (15%) were annotated to plasma membrane where 51 and 38 genes were upregulated while 244 and 143 were downregulated, respectively (Figure 4.8). For chloroplast, upregulated genes including 2-cys peroxiredoxin 1, peroxiredoxin and 20 kDa chaperonin upregulated were involved in detoxification and stabilisation of proteins. These genes possess roles that may provide protection to the cells against damage by chilling stress[303,304] and their upregulation in response to chilling treatment may represent a response to damage caused by chilling.

Genes such as glutamyl t-RNA reductase involved in chlorophyll biosynthesis[299] and ribulose-1,5-bisphosphate carboxylase playing a crucial role in photosynthesis were downregulated. Thylakoid luminal proteins such as PsaF, PSB27 and monogalactosyldiacylglycerol synthase were also downregulated where PSB27 protects PSII from photodamage[305] and mongalactosyldiacylglycerol is involved in

thylakoid membrane biogenesis[306]**.** Biological information of chloroplast associated downregulated genes, suggest that photosynthesis, thylakoid membrane, and chlorophyll biosynthesis were most probably affected as a result of chilling stress.

Genes annotated to plasma membrane, including gamma-Tip, heat shock 70kDa and a sugar transporter that are involved in facilitating water transport, stabilisation of proteins and distribution of sugar around the cell[279,307,308], were upregulated and may possibly represent cellular response to damage by chilling.

Various genes involved in calcium signalling which are important for cold acclimation, such as calcineurin-B like protein 10, calcineurin-B like protein 3, calcium-transporting ATPase, and a MAPK, were downregulated[53]. This finding suggests that basil shows a downregulation of calcium signalling rather than the ability to amplify its calcium signalling that is needed for cold acclimation and this perhaps could also account for its inability to tolerate chilling temperatures.

The findings of this present study are also compatible with those of Zhan *et al.*[272] where cell and cell part were identified as the most enriched cellular components in gene expression changes in *O. Americanum* var. pilosum basil during chilling stress. According to the gene ontology consortium, cell is defined as "all components within and including the plasma membrane and any external encapsulating structures, such as the cell wall and the cell envelope" whereas cell part is defined as "any constituent part of a cell, the basic structural and functional unit of all organisms". Hence, while Zhan *et al.*[272] identified parent terms, cell and cell part, to be the most enriched during chilling stress, this present study has identified the more specific child terms of chloroplast and plasma membrane to be the cellular components most affected by chilling temperature.

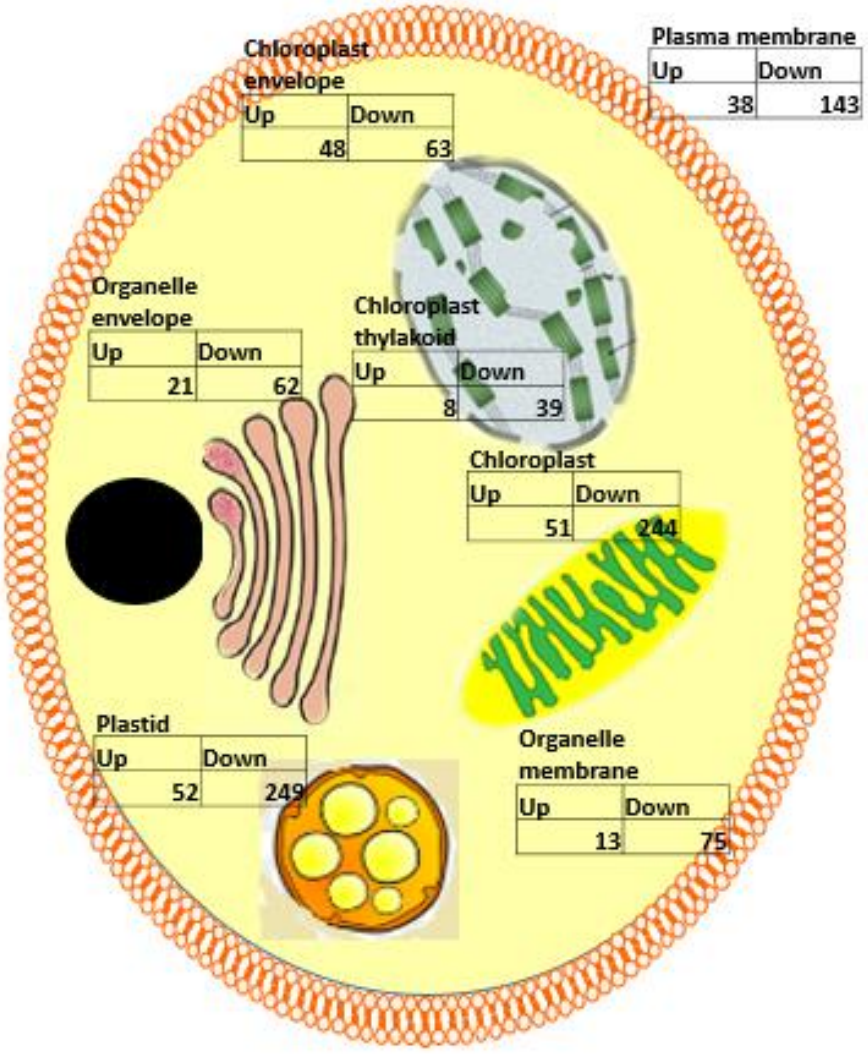


Figure 4.8 Cell and cellular components identified as significantly over-represented (P value < 0.05 based on Fisher's exact p value by EASE) among differentially regulated genes in basil in response to cold stress.

4.3.6.4 Molecular functions that are overrepresented among differentially regulated genes:

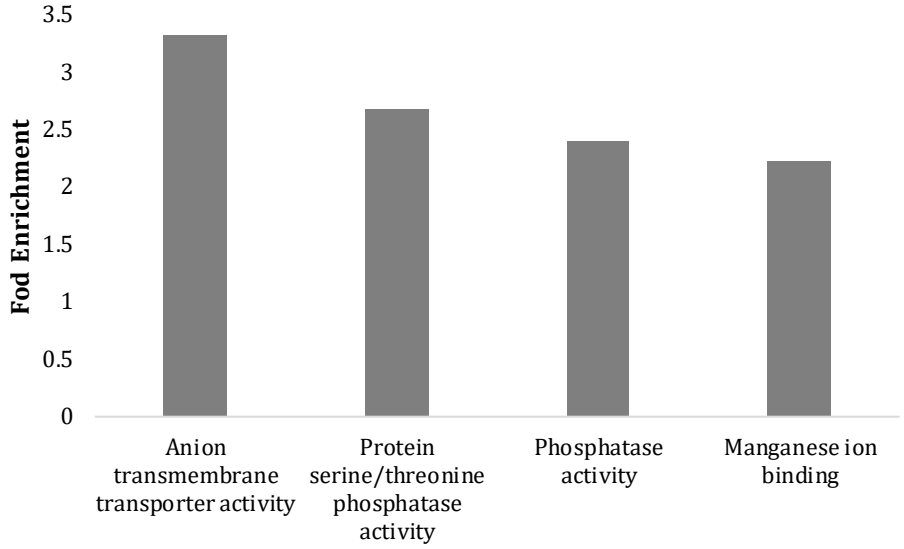


Figure 4.9 Molecular functions of genes significantly overrepresented (P value < 0.05 based on Fisher's Exact p value by EASE) among differentially regulated genes identified by the DAVID functional annotation tool.

The molecular functions enriched among differentially regulated genes include transporter activity of anions, phosphatase activity and manganese ion binding, identifying them as likely to be relevant in basil's cellular response to chilling temperatures (Figure 4.9) (details of genes annotated to each molecular function are provided in appendix 4.4). These findings are similar to those of Zhan *et al*.[272] where parent terms such as transporter activity, catalytic activity and binding were identified as enriched. These molecular functions were particularly found to be associated with genes that were downregulated in response to chilling conditions. Genes annotated as being involved in transporter activity associated with the plasma membrane; phosphatase activity associated to cytosol or chloroplast; and magnesium ion binding associated to cytosol were overrepresented among downregulated genes. Downregulation of genes with the function of transportation of ions suggests that the distribution of ions across the cell could be possibly reduced and thereby could cause cellular ion homeostasis to be disturbed.

The genes coding for phosphatase activity were associated to the stroma of the chloroplast. Stromal enzymes are involved in the calvin cycle and chilling stress led to inhibition of these enzymes in tomato that resulted in photosynthetic inhibition in chilling sensitive tomato[309]. Hence, it may be possible that downregulation of genes coding for proteins with photosynthetic activity may also play a role in basil chilling sensitivity.

4.3.7 KEGG pathway analysis

To identify biochemical pathways that are overrepresented as active basil exposed to chilling conditions, the differentially regulated genes were also subjected to Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis. The KEGG pathway analysis revealed that the three most significantly overrepresented pathways (P value < 0.05 based on Fisher's Exact p value by EASE) were arginine and proline metabolism, the pentose phosphate pathway and carotenoid biosynthesis (details of genes annotated to each pathway are provided in appendix 4.5) that are child terms of parent terms carbon metabolism and amino acid biosynthesis identified by Zhan *et al.*[272]

The proline metabolism and carotenoids biosynthesis metabolic pathways enrichment among differentially regulated genes in response to cold is of interest to find, as both the pathways have previously been shown to promote cold tolerance in plants[129,156,170,310].

The arginine and proline metabolism pathway was annotated to genes including arginase 1, glutamine synthetase and pyrroline-5-carboxylate reductase. These genes are involved in proline accumulation[311–313] but were found downregulated in basil's response to cold.

Proline and raffinose play roles in water deprivation stress and oxidative stress due to their osmoprotectant and antioxidative properties[314]. Proline and raffinose are

induced in plants at times of environmental stresses and associated with increased tolerance of plants to salt stress, drought stress and cold stress[310,315,316]. Osmolytes such as proline and raffinose balance the osmotic difference between the inside and outside of the cell to prevent the loss of water from the cell, reduce oxidative stress and also serve as a carbon source to resume growth in plants after the stress is removed[314,317]. Proline appears to target the symptoms commonly observed during chilling stress in sensitive plants namely water loss from cells, oxidative stress and suppression of growth. Accumulation of proline led to increased cold tolerance in chilling sensitive maize callus and may also possibly increase a chilling sensitive plants ability to tolerate low temperatures. Hence, downregulation of genes involved in proline accumulation is likely to be disadvantageous for basil plants and may contribute to their susceptibility to damage by chilling conditions.

Carotenoids content was found increased in *Zea mays* plants grown under chilling conditions[156]. A higher photosynthetic activity in chilling tolerant genotype was also attributed to higher accumulation of carotenoids content in tolerant *Zea mays* than in the sensitive ones[156]. Overexpression of chyB gene involved in the zeaxanthin biosynthesis led to increased size of xanthophyll cycle pool[129]. *A. thaliana* overexpressed with chyB gene showed increased tolerance to oxidative damage from high light and high temperature stress[129]. Hence, it is likely that a possibly increased content of carotenoids in basil may also increase its tolerance to oxidative stress in chilling conditions. Enrichment of metabolic pathways such as proline metabolismand carotenoid biosynthesis from differentially expressed list of genes indicates that basil is a responsive plant to chilling stress and is able to induce molecular changes relevant to development of chilling tolerance.

4.3.8 The visualisation of chilling temperature's effect on primary metabolism of basil exposed to chilling conditions:

The effect of chilling temperature on primary metabolism, that includes metabolic pathways essential for the survival and functioning of the cell, was investigated using Mapman software. Mapman allows for visualisation of differentially expressed genes overlaid on diagrams of metabolic pathways.

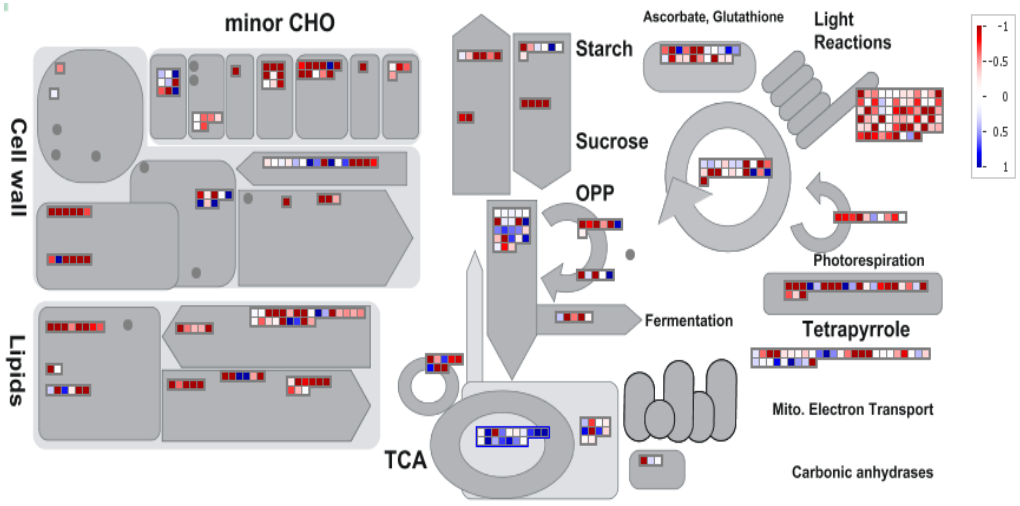


Figure 4.10 Shows output from Mapman software depicting the effect of chilling temperature on primary metabolism of the cell in basil. The differential expression of genes was coded by colour and represented on a colour gradient log 2 scale of -1 to +1 where red represents downregulation of genes. (-1 represents at least 0.5 fold change) while blue represents upregulation (+1 represents at least 2 fold change).

The visualisation of primary metabolism (Figure 4.10, Table 4.2) shows that the various different metabolic pathways were predominantly downregulated. Lipid metabolism, carbohydrate metabolism including light reactions, Calvin cycle, sucrose and starch metabolism and ascorbate and glutathione antioxidant defence system were mostly downregulated while glycolysis and pyruvate cycle were upregulated.

Fatty acids make up the back bone of phospholipids in the cellular membrane and are also useful as an energy source. One of the common mechanisms employed by the plant to combat injury by chilling stress is to bring changes in lipid composition of the

membrane to protect its integrity and stability during low temperature stress[318]. This involves reprogramming of lipid metabolism that includes increased desaturation of fatty acids by desaturase enzymes.

Lipid synthesis in the basil transcriptomic profile appears to be predominantly downregulated after treatment with chilling temperature. Key genes involved in lipid synthesis such as phosphatidylglycerolphosphate synthase 2 (PGPS2) and monogalactosyl diacylglycerol synthase 1 (MGD1) were downregulated as 0.2 and 0.5 fold, respectively. This finding is similar to those of study by Hannah *et al.*[141] where *A. thaliana* exhibited downregulation of lipid metabolism in response to short (12 h), medium (48 h) and long (120 h) term exposure to chilling stress. The genes involved in fatty acid synthesis, elongation, synthesis of phospholipids and degradation by lipases and lysophospholipases were all downregulated in response to chilling stress. The downregulation of lipid metabolism was proposed by Hannah *et al.*[141] to be due to reduced demand for growth and development at low temperature. Similarly, basil downregulation of genes involved in phospholipid synthesis suggests that growth of cells may be reduced as an initial response to chilling temperature. Reduced growth is often observed in chilling sensitive plants and is important for adaptation to low temperature of the environment. Reduced growth rate prevents utilisation of energy substrates so that they can be used to provide cryoprotection to cell against injury by chilling stress[319].

However, the desaturases upregulated in *A. thaliana* and associated with acclimation to cold[320] were found downregulated in basil. The downregulation of FAD2 and FAD5 indicates that basil, similar to other chilling sensitive plants, is likely not able to provide protection to its cell membrane against damage by chilling temperature.

It seems that downregulation of lipid synthesis and degradation could occur in basil to adjust the metabolism to low temperatures and may even be compatible with cold

acclimation. However, this reprogramming of metabolism in response to chilling conditions does not necessarily exclude the possibility that reduced lipid synthesis can also result in less substrate being available for recovery of damaged membranes by chilling conditions[186]. In addition, downregulation of desaturases that could not only introduce double bonds in newly synthesised lipids but also cause desaturation of fatty acids existing in the membranes, likely renders membranes susceptible to reduced fluidity damage commonly induced by chilling conditions[321].

Carbohydrates accumulation in plants is yet another common mechanism that is associated with increased tolerance of the plants towards low temperatures. High amounts of carbohydrates can provides cryoprotectant, osmolyte, scavenging of ROS and signalling molecule effects to the cell[107,174]. In order for carbohydrates to be generated and maintained in the cell at high quantities, inhibition of photosynthesis under chilling conditions must be prevented. At chilling temperatures, phloem export is inhibited in *A. thaliana* that causes carbohydrates to accumulate in the leaves and results in downregulation of photosynthetic genes[322]. Downregulation of photosynthesis at chilling temperatures was also found in chilling tolerant *A. thaliana*[141]. However, upon cold acclimation the chilling tolerant plants such as spinach, rye and wheat showed remarkable recovery of photosynthetic capacity by increasing activation of enzymes involved in Calvin cycle such as ribulose bisphosphate carboxylase/oxygenase activase (RuBisco activase) and fructose-bisphosphate aldolase[323–325]. In basil, genes involved in light reactions, the Calvin cycle and photorespiration showed downregulation; thus, in this respect basil behaved more like *A. thaliana.* For example, expression of genes coding for RuBisco activase and fructose-bisphosphate aldolase were found downregulated in chilling sensitive basil. Thus, basil may be displaying some adaptation to chilling.

An alternative route to cope with the imbalance of source and sink other than changing activity of enzymes involved in the Calvin cycle, glycolysis and TCA cycle is reduction in the size of the antenna system[326]. The downregulation of genes involved in tetrapyrrole synthesis observed in basil's transcriptomic profile under chilling conditions, therefore, may have occurred to ensure that the plants absorb less light energy to prevent photooxidative damage from occurring on photosystems and to prevent photosynthesis from undergoing photoinhibition. Again, this suggests that basil may be displaying some adaptation to chilling.

Genes involved in starch synthesis and starch degradation were also supressed including downregulation of genes such as starch synthase 2 (SS2), SS3, glucose-1-phosphate adenylyltransferase (APL4) and beta-amylase (BAM9). Analysis of starch metabolism in various different plant species has shown its response to chilling temperatures to be species specific. Starch synthesis remains unaffected or even increases in cold tolerant plants such as *A. thaliana*, cabbage and sugar cane but starch declines in its content and its synthesis in chilling sensitive species[46,327,328]. It therefore appears that starch synthesis may not be negatively affected in chilling tolerant plants but may not necessarily play an important role in chilling tolerance. Basil, therefore, behaves like other chilling sensitive species in this respect.

The ascorbate and glutathione cycle forms part of primary metabolism that protect the cell from adverse effects of accumulated H_2O_2 at times of chilling stress[329]. Zea mays showed increased glutathione pool that was accompanied with better tolerance towards chilling temperatures by causing detoxification of free radicals[330]. Basil genes involved in ascorbate and glutathione cycle exhibited low gene expression with monodehydroascorbate reductase (MDAR4), glutathione peroxidase 5 (GPX5) and glutathione peroxidase (GPX6) as downregulated. These findings suggest that basil is

likely to suffer from reduced expression of enzymes involved in prevention from damaging effects of excess ROS generated at times of chilling stress.

The basil transcriptomic profile show symptoms of chilling damage that is typically observed in chilling sensitive plants that includes greater downregulation of genes than upregulation. Chilling sensitive plants in response to chilling temperature exhibit cessation of growth, suppression of chlorophyll synthesis, downregulation of photosynthetic genes, reduced sucrose synthesis, sucrose degradation and starch synthesis that were all observed in basil's primary metabolism transcriptomic profile.

While there is reprogramming of metabolism to prevent the cell from damage posed by chilling stress, by causing reduced lipid and chlorophyll synthesis that may even be an adaptive approach temporarily, this strategy is, however, clearly not sufficient to provide appropriate tolerance to basil cells over the period of 24 h of chilling stress. The changes occurring in genes involved in primary metabolism may result in basil cells having less substrate to replace damaged membrane components, lack of protection against deleterious effects from ROS, and less accumulation of carbohydrates that provide cryoprotective effect to the cell against injury by low temperature. The cells are also likely to suffer from reduced growth due to reduced photosynthesis in response to reduced expression of photosynthetic genes involved in the light cycle and the Calvin cycle. According to Lukatkin *et al.*[87] is these accumulating damaging effects to the cell and cellular components that leads chilling injury to become visually apparent on tissues of chilling sensitive plant.

Primary metabolic pathway	Tair_ID	Official gene symbol	Log_2 Change
Lipid metabolism.Phospholipid synthesis	AT4G00400	GPAT8	-2.1481
Lipid metabolism.Phospholipid synthesis	AT1G01610	ATGPAT4	-1.82236
Lipid metabolism.glycolipid synthesis.MGDG synthase	AT4G31780	MGD1	-1.00313
Lipid metabolism.FA desaturation.desaturase	AT3G15850	FAD5	-1.1819
Lipid metabolism.FA desaturation.omega 6 desaturase	AT3G12120	FAD2	-1.63138
Lipid metabolism.FA synthesis and FA elongation.acyl coa ligase	AT5G23050	AAE17	-1.52083
PS.lightreaction.photosystem II.LHC-II	AT1G76570	28729132-28730825 FORWARD	-1.79176
PS.lightreaction.photosystem II.LHC-II	AT1G15820	LHCB6	-1.24158
PS.lightreaction.photosystem II.PSII polypeptide subunits	AT4G28660	PSB28	-0.29474
PS.lightreaction.photosystem II.PSII polypeptide subunits	AT1G03600	898876-899655	-2.48615
PS.lightreaction.photosystem I.LHC-I	AT1G45474	LHCA5	-1.25634
PS.lightreaction.cytochrome b6/f	AT5G36120	CCB3	-1.16993
PS.lightreaction.cyclic electron flow-chlororespiration	AT2G05620	PGR5	-1.80829
Glycolysis.cytosolic branch.fructose-2,6-bisphosphatase (Fru2,6BisPase)	AT1G07110	F2KP	-1.17996
Glycolysis.cytosolic branch.non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase (NPGAP-DH)	AT2G24270	ALDH11A3	-2.18797
Glycolysis.cytosolic branch.phospho-enol-pyruvate carboxylase (PEPC)	AT1G68750	ATPPC4	-1.32376
Redox.ascorbate and glutathione	AT4G18260	10093524-10097503 REVERSE	-2.14684
Redox.ascorbate and glutathione.ascorbate	AT3G27820	ATMDAR4	-1.64997
Redox.ascorbate and glutathione.ascorbate	AT1G75270	DHAR2	-1.05247
Redox.ascorbate and glutathione.glutathione	AT3G63080	ATGPX5	-1.88583
Redox.ascorbate and glutathione.glutathione	AT4G11600	ATGPX6	-1.05063

Table 4.2 Differentially expressed genes in response to chilling conditions that are involved in the metabolic pathways of primary metabolism.

4.3.9 Genes of basil most responsive to chilling temperature:

TAIR_ID	Gene symbol	Description	Expression fold difference
AT1G71100	RPI1	Probable ribose-5-phosphate isomerase	25.80555556
AT437640	ACA2	Calcium-transporting ATPase 2, plasma membrane-ty	15.07142857
AT1G78370	GSTU20	Glutathione S-transferase U20	12.355
AT5G40010	AATP1	AAA-ATPase	11.70852018
AT3G05500	N/A	REF/SERP-like protein	10.45985401
AT4G38740	CYP18-3	Peptidyl-prolyl cis-trans isomerase CYP18-3	9.27184466
AT5G54940	N/A	Translation intiation factor SUI1 family protein	7.204819277
AT3GG3460	CP29A	29 kDa ribonucleoprotein, chloroplastic	7.204819277
AT3G48990	AAE3	Oxalate-CoA ligase	7.204819277
AT2G16600	CYP19-1	Peptidyl-prolyl cis-trans isomerase CYP19-1	7.033816425
AT2G22910	NAGS1	Probable amino-acid acetyltransferase NAGS1, chloroplastic	0.0313
AT4G14740	N/A	Plant protein of unknown function (DUF828) with plant pleckstrin homology-like region	0.0504
AT4G11650	OSM34	Osmotin-like protein OSM34	0.0566
AT1G51340	MATE	MATE efflux family protein 1	0.0802
AT5G20220	N/A	zinc knuckle (CCHC-type) family protein	0.0816
AT3G22810	N/A	Plant protein of unknown function (DUF828) with plant pleckstrin homology-like region	0.0842
AT2G17650	AAE2	Probable acyl-activating enzyme 2	0.0856
AT4G08570	N/A	Heavy metal transport/detoxification superfamily protein	0.0878
AT2G14750	APK1	Adenylyl-sulfate kinase 1, chloroplastic	0.0909
AT3G21260	GLTP3	Glycolipid transfer protein 3	0.0972

Table 4.3 TAIR ID, official gene symbol, description and relative differential gene expression of most responsive genes to chilling temperature.

The genes that exhibited the largest response to chilling temperatures may be of interest therefore details of top 10 most up and down-regulated genes were studied (Table 1.3). The majority of these top few differentially regulated genes were annotated to be associated to chloroplast, cytosol and membrane. From the top ten up-regulated genes, the ones whose roles are well known include ribulose-5-phosphate isomerase, calcium-transporting ATPase, glutathione S transferase, cis-trans isomerase CYP18-3, CYP-19 and 29kDa ribonucleoprotein that are involved in the Calvin cycle, calcium signalling, detoxification of ROS and stabilisation of proteins at times of abiotic stress, respectively[61,331–333]. These activities can provide protective effects to the cell from damaging effects induced during chilling stress and have already been discussed above. From the top 10 downregulated genes (Table 1.3) there are a few such as Mate efflux, acyl activating enzyme that provide ROS scavenging roles, zinc knuckle (CCHC) family protein which is a probable protein heat-shock protein type chaperone and osmotin that provides tolerance towards dehydration and salt stress whose downregulation is not compatible with cold acclimation[334–336]. The acetyltransferase, N-ACETYL-L-GLUTAMATE SYNTHASE 1, is involved in amino acid biosynthesis so its decrease is consistent with the observed overall decrease in amino acid biosynthesis genes.

Functions for the remaining genes on the up and down regulated lists have not previously been identified and, therefore, this demonstration that they are dramatically affected by chilling may give functional clues that are useful to other labs working on these classes of genes. Of particular interest are the two genes in this category that are predicted to encode proteins containing plekstrin-like domains. The occurrence of these two very similar genes within the top 10 most downregulated genes may suggest that this class of genes has particular importance in the basil cold response.

4.3.10 CBF transcription factors and their existence in basil.

The CBF transcription factors of the cold regulatory pathway were not found in the RNA sequencing transcripts database of this study. However, CBF1, CBF2 and CBF3 were identified in the study by Zhan *et al.*[272] and their low expression in response to chilling temperature was claimed to be the cause of basil's inability to tolerate chilling temperatures. Zhan *et al.*[272] showed that CBF1, CBF2 and CBF3 showed 33.33%, 52.38% and 36.78% percentage identity, respectively to orthologues from tomato.

In this study we only consider the query sequence and the subject sequence to be orthologous when they meet the criteria of bit score of at least 80 and e value of 10^{-10} so that only sequences with significant homology were considered for further investigations such as functions and their involvement in biological processes. It is then possible that due to the stringent conditions used, the identification of CBFs may have possibly been filtered out (type II error). It may be possible that due to the evolutionary distance across organisms being more conserved at amino acid sequence level rather than at nucleotide level, the CBF genes may have possibly been picked and identified had the amino acid sequences were aligned rather than nucleotide sequences. The use of amino acid sequences for alignment also allows the user to use less stringent conditions such as bit score of 50 rather than 80 to be able to infer homology with the output sequence[200]. This would further increase the chances of being able to pick out more sequences that may have otherwise been filtered out in a nucleotide sequence alignment.

4.4 Conclusion:

The enrichment analysis has facilitated our understanding in the biological processes, cellular components and molecular functions that are of relevance in basil's cellular response to chilling conditions. It was through these enrichment analysis that three clusters of biological processes were identified which showed enrichment among genes upregulated in response to cold in basil: cluster 1 (response to cold, response to temperature stimulus and response to abiotic stimulus), cluster 2 (response to abiotic stimulus, response to salt stress and response to osmotic stress) and cluster 3 (response to cadmium ion, response to metal ion and response to inorganic substance). Genes within these clusters included Arginine decarboxylase 1, arginine decarboxylase 2, heat shock cognate 70 kDa protein, heat shock protein 81-2, 2-Cys peroxiredoxin BAS1,aconitate hydratase, fructose-bisphosphate aldolase and peroxiredoxin and these are, therefore, potentially important for further investigation.

In addition genes involved in chlorophyll biosynthesis, response to light stimulus, photo protection of photosystems and detoxification of ROS were downregulated. Differences in the regulation of these processes between chilling tolerant versus chilling susceptible plants suggests that their response patterns may be important in basil being susceptible to injury by chilling conditions. It appears that basil under chilling conditions may arrest its growth and development as evidenced by the reduced expression of genes involved in primary metabolism. Chloroplast and plasma membrane components appear to play important roles in basil cellular responses to chilling conditions as these cellular components were enriched among genes showing downregulation in response to cold. There was downregulation of genes coding for various proteins including desaturases, proteins involved in calcium signalling, sugar transportation, and antioxidative enzymes with role in scavenging of ROS. Possibly

most importantly, there appeared to be a lack of genes coding for CBF transcription factors but this could be due to the stringent conditions used in identifying transcripts that bear homology with basil RNA seq transcripts.

In summary, the defence mechanisms commonly employed by chilling-tolerant plants to combat against chilling stress appeared to be supressed or absent in basil. There was a lack of response among genes associated with the protective mechanisms against chilling induced photooxidative damage to photosystems, inhibition of photosynthesis, rigidification of membrane, and accelerated levels of ROS which could have led basil to show damaging symptoms on its tissues upon exposure to chilling temperature for 24 h. Thus, this pre-screening of basil can provide valuable information to be further investigated and used in developing strategies to improve chilling tolerance in chilling sensitive Marian basil.

The enriched biological process by differentially regulated genes have shown that the genes coding for antioxidative enzymes to play important role in basil's response to chilling stress. Hence, manipulating metabolic pathways such as inducing genes involved in carotenoids biosynthesis, ascorbate and glutathione cycle would be worth investigating further, as this would give an indication of the importance of antioxidative enzymes' role in improving basil's ability to tolerate cold temperatures.
The enriched biological process by differentially regulated genes have shown that the genes coding for antioxidative enzymes to play important role in basil's response to chilling stress. Hence, manipulating metabolic pathways such as inducing genes involved in carotenoids biosynthesis, ascorbate and glutathione cycle would be worth investigating further, as this would give an indication of the importance of antioxidative enzymes' role in improving basil's ability to tolerate cold temperatures.
Transgenic basil plants carrying gain-of-function mutation to overexpress arginine

decarboxylase 1 and arginine decarboxylase 2 involved in the synthesis of polyamines can be created ad their effect on basil chilling tolerance tested.

The molecular changes observed in basil's response to chilling conditions show that chilling temperature has a globally suppressive effect on basil's transcriptome. In addition, chlorophyll biosynthesis has previously been discussed as important for chilling tolerance but was found downregulated in basil in response to cold. Decline in chlorophyll biosynthesis has also been observed in other chilling sensitive plants. Thus, downregulation of chlorophyll biosynthetic genes in basil is not surprising and indicates its importance in basil's sensitivity to chilling temperatures. These findings indicate that the use of light treatment in inducing chilling tolerance in basil may be a useful one. Use of a light treatment may be able to reverse the downregulation of chlorophyll biosynthetic genes and support basil's acclimation to cold temperatures.

Accumulation of proline in cells can combat with the three main symptoms (oxidative stress, water loss, growth retardation) normally induced by chilling stress in chilling sensitive plants. Hence, downregulation of genes involved in proline accumulation in basil under chilling stress could explain basil's inability to tolerate chilling stress. On the other hand, alpha glucan water dikinase involved in starch degradation and accumulation of sugars in *A. thaliana* was found upregulated in basil after storage at chilling temperature. Alpha glucan water dikinase increased expression in *A. thaliana* led to increased freezing tolerance and therefore increased expression of alpha glucan water dikinase could have provided some protection to basil from injury by chilling stress. Thus, to improve basil tolerance towards chilling conditions, a treatment was required that was able to induce expression of genes involved in scavenging of ROS, accumulation of sugars and reducing cellular water loss. Loss of phytochromes in *A. thaliana* (*phyBD, phyABDE*, and *phyABCDE*) led to elevated levels of sugars and proline but the greatest impact was observed in *phyB* and *phyD* mutants [337]. These findings

suggests that active phyB and phyD are likely to have a suppressive effect on accumulation of proline and thereby reduce the development of cold tolerance in plants. Light that is low in red to far red ratio (LR:FR) cause inactivation of phyB and may thereby allow accumulation of proline and sugars needed for cold acclimation in plants. In addition, LR:FR light can cause increased expression of ABA[261] that increases plants tolerance to water loss during chilling stress[168]. Thus, LR:FR light may be a suitable treatment in inducing chilling tolerance in sensitive basil as this treatmentmay be able to induce expression of genes that were otherwise found downregulated in basil under chilling stress but are needed for cold acclimation in basil.

5 EFFECT OF LIGHT QUALITY ON THE CHILLING TOLERANCE ABILITY OF BASIL VAR MARION

5.1 Introduction

5.1.1 The suitability of lr:fr light treatment in inducing chilling tolerance in chilling sensitive basil:

The RNA-sequencing analysis from Chapter 4 showed that chilling stress causes global molecular changes in basil cells that may contribute to its sensitivity towards chilling conditions. Hence, light treatment was chosen as an environmental factor to improve chilling tolerance in basil, as light has profound effects on the plant's growth and development[338] and causes activation of various cold-responsive genes[182,296]. The light regime described by Franklin and whitelam[183] was used in this present study to try and improve chilling tolerance in sensitive basil. The light regime involved treating basil with LR:FR light from ZT4-ZT8 for four days at ambient temperature and in 12 h photoperiod. The light regime described by Franklin and Whitelam[183] proved effective in inducing freezing tolerance in *A. thaliana* at temperatures higher than those required for cold acclimation. *A. thaliana* was grown at 16^0C and showed a survival rate of only 37% after treatment at 0^0C. However, when *A. thaliana* was treated with LR:FR light from ZT4-ZT8 for four days prior to its storage at 0^0C, the survival rate increased and reached up to 87%, indicating that LR:FR light was an effective treatment in inducing freezing tolerance in this freezing sensitive plant[183].

According to Franklin and Whitelam[183], the development of increased freezing tolerance in *A.thaliana* was a result of LR:FR light-mediated inactivation of phyB and phyD from Pfr to Pr. Treatment of *A. thaliana* with LR:FR light between ZT4 and ZT8 may cause inactivation of phyB at the time points when PIF4 and PIF7 expression are likely to peak[177]. Hence, it is likely that inactivation of phyB by LR:FR light may prevent PIF4 and PIF7 from interacting with phyB and allow CBF genes to be expressed without

repression from phyB, PIF4 and PIF7 between ZT4-ZT8. CCA1 and LHY stimulate the expression of CBF genes from ZT3 onwards and the CBF gene family peak in their expression at ZT8[65]. So, irradiating *A. thaliana* with LR:FR light from ZT4-ZT8 will prevent repression on CBF genes for the entire time that CBF genes can be expressed in the plant. Lack of repression from ZT4-ZT8 will allow CBF genes to be expressed at a higher rather than normal and thereby provide the plant with greater cold tolerance. Thus, in this chapter the effect of LR:FR light at inducing chilling tolerance in chilling sensitive basil was investigated.

5.1.2 Aquaporins role in chilling tolerance of plants:

Other than the physiological damage experienced by the cell and the downregulation of various genes involved in basil metabolism, one chilling injury symptom that was commonly observed in basil on its exposure to 24 h of chilling stress was the wilting of the leaves. According to Yu *et al.*[146] wilting of the leaves is a result of water imbalance occurring in cells at times of chilling stress. The water imbalance occurs in response to reduced water uptake from the roots and transpiration from the leaves during chilling stress[146]. Aquaporins are water channel proteins located on the membrane of the cell or the vacuole that allow passive movement of water molecules across the membrane[339]. Aquaporins have shown differential gene expression in response to chilling and drought stress and therefore have been suggested to play roles in plant response to abiotic stress related to water deficit including chilling, salt and dehydration stress[340]. Hence, this Chapter not only focuses on determining the effect of LR:FR light on cellular damage induced by chilling temperatures but also the effect of different light regimes and chilling temperatures on the water status of basil cells.

5.2 Results and Discussion

The aim of this chapter was to test the proposal that periodic treatment with LR:FR light during the middle part of the day could induce chilling tolerance in basil. Twenty-nine day old adult plants ready for market were grown in 12 h HR:FR light and 12 h dark cycles for 4 days. Control plants were maintained in these conditions throughout, while experimental plants were treated with periodic LR:FR light via supplementation with additional FR for 4 hours from ZT4 – ZT8 on each of the 4 days. The pots were then exposed to chilling temperature after which phenotypic assessment was carried out.

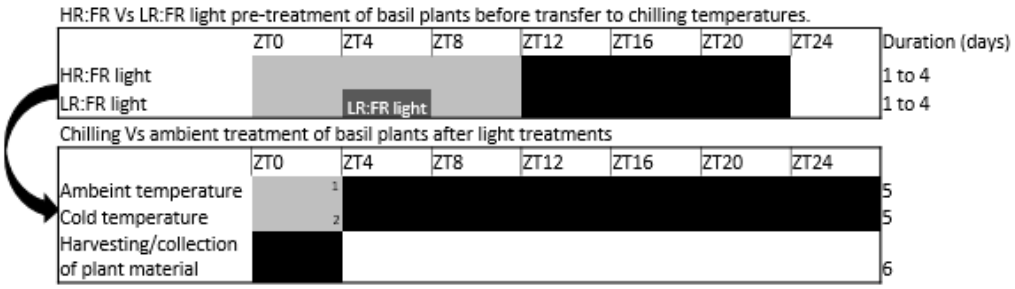


Figure 5.1 Basil plants were either maintained in HR:FR light or pre-treated with periodic LR:FR light (ZT4-8) for four days at 12 h photoperiod. Basil plants were subsequently stored in either ambient or chilling temperatures for 24 h under continuous dark conditions on the fifth day of the experiment. Samples of basil were harvested on the sixth day at ZT4 from ambient or chilling temperature (1) and (2).

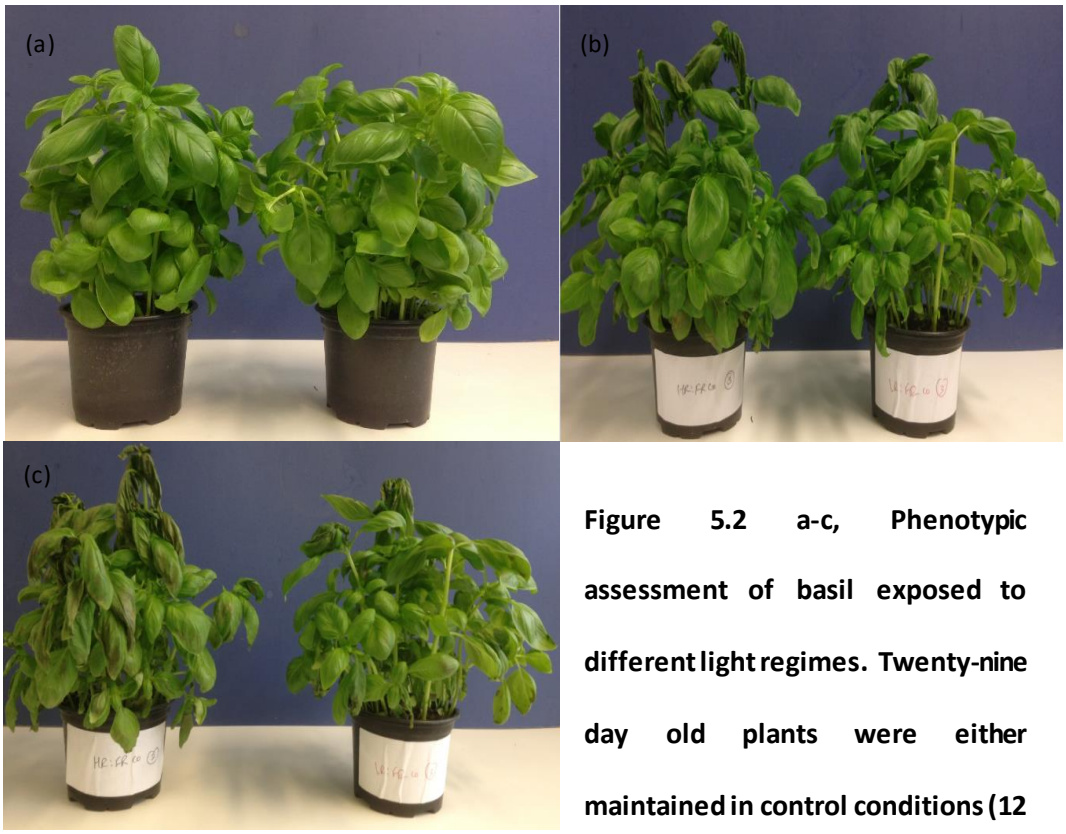


Figure 5.2 a-c, Phenotypic assessment of basil exposed to different light regimes. Twenty-nine day old plants were either maintained in control conditions (12 h HR:FR light: 12 dark) for 4 days (left, in each image) or treated with periodic LR:FR light (right, in each image) on each of the 4 days. The pots were then exposed to ranging from 0 h (a), 12 h (b) to 24 h (c) of chilling temperature. Each figure is representative of 7 pots per time point.

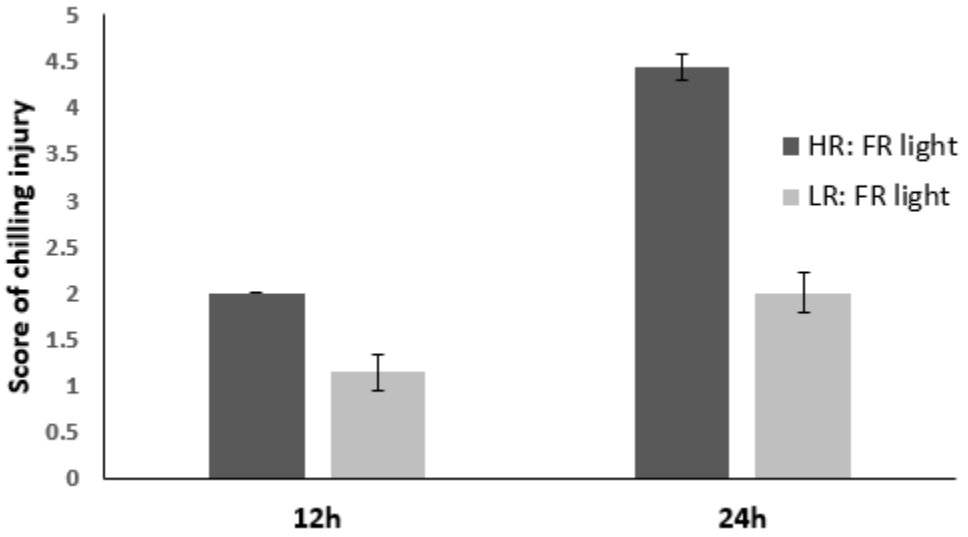


Figure 5.3 Score of chilling injury from phenotypic assessment of basil pots exposed to 12 h or 24 h of chilling temperature (4^0C), after treatment with or without periodic LR:FR light for four days. Pots of basil were subsequently photographed and scored according to the degree of chilling damage the pots of basil displayed. Each value represents the mean ± SE where n = 7.

Pots of basil irradiated with either of the two different light regimes (either maintained in control HR:FR or given periodic treatment with LR FR light) before having been exposed to varying durations of chilling stress were graded according to the injuries observed on their tissues. Following 24 h of chilling treatment, basil pots maintained in HR:FR light showed much greater development of chilling damage than LR:FR light treated plants. This damage involved strong wilting of the leaves, and discoloration on more than 50% of the leaf area (Figure 5.2C). Thus, basil maintained in HR:FR light and then subsequently exposed to chilling conditions for 24 h was graded with an average score of 4.42 out of 5 for damage (See chapter 3, results and discussion for details of the scoring system) whereas basil treated with LR:FR light and subsequent exposure to 24 h of chilling stress was graded with an average score of 2 for displaying wilting of the leaves only (Figure 5.2C and 5.3). From these visual analyses it appears that the

LR:FR light regime had caused basil to develop significantly less chilling damage and therefore appears to be effective in enhancing the tolerance to chilling conditions.

5.2.1 Effect of LR:FR light quality on basil's cell membrane stability following its exposure to chilling stress:

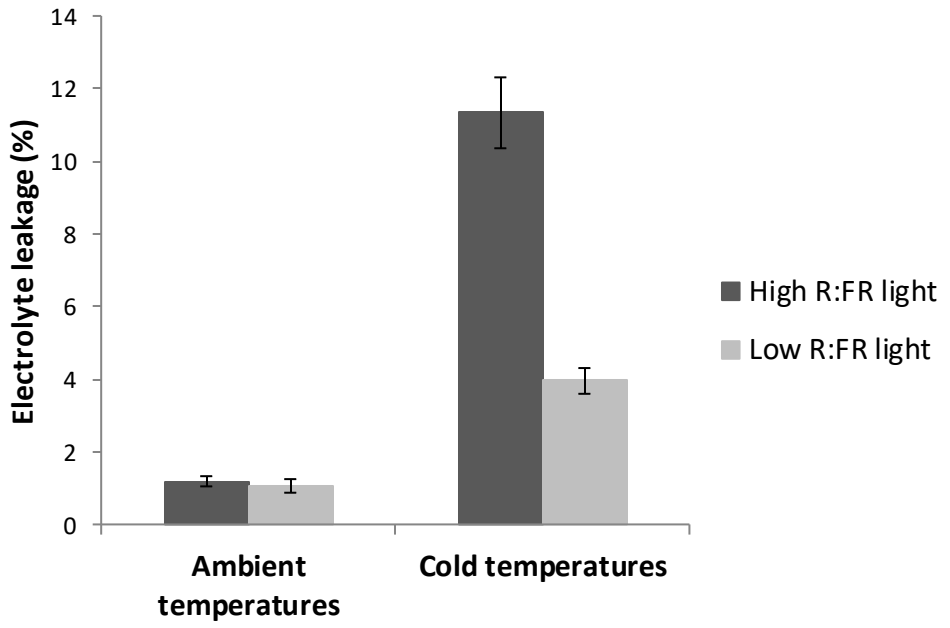


Figure 5.4 Electrolyte leakage from leaves of 29-day old basil plants either maintained in control HR:FR light or treated with periodic LR:FR light at ambient temperatures with a 12 h photoperiod for 4 days prior to exposure to either continuous darkness ambient or continuous darkness chilling conditions (4°C). Each bar represents mean ± SE and n = 10.

The results from electrolyte leakage measurements showed that there was a statistically significant difference between groups as determined by Welch ANOVA ($F(3, 36) = 82.062$, $p =< .001$)[4]. The Games-Howell post hoc test revealed that there was a statistically significant difference between basil plants treated at ambient temperatures and those treated at chilling temperatures when maintained in HR:FR

[4] There was heterogeneity of variance between the samples. As a result, Welch ANOVA and Games Howell post hoc test was used, as they are the most robust test that can compare the two means effectively despite the inequality of variances between populations [451].

(*p=.=< .001)* or pre-treated with LR:FR light (*p=0.004)* (Figure 5.4). The Games-Howell post hoc test also revealed that there was a significant difference between chilled basil that was maintained in HR:FR or pre-treated with LR:FR light *(p=< .001).* Basil pre-treated with LR:FR light displayed significantly lower electrolyte leakage after storage at chilling temperature for 24 h than basil maintained in HR:FR light by 65% (Figure 5.4). The electrolyte leakage results indicated that basil pre-treated with LR:FR light to have suffered less membrane damage when exposed to chilling temperatures and, therefore, pre-treatment with LR:FR is likely to be effective in providing tolerance to basil against damage by chilling temperatures.

5.2.2 Effect of LR:FR light on the accumulation of ROS in basil leaves exposed to chilling stress:

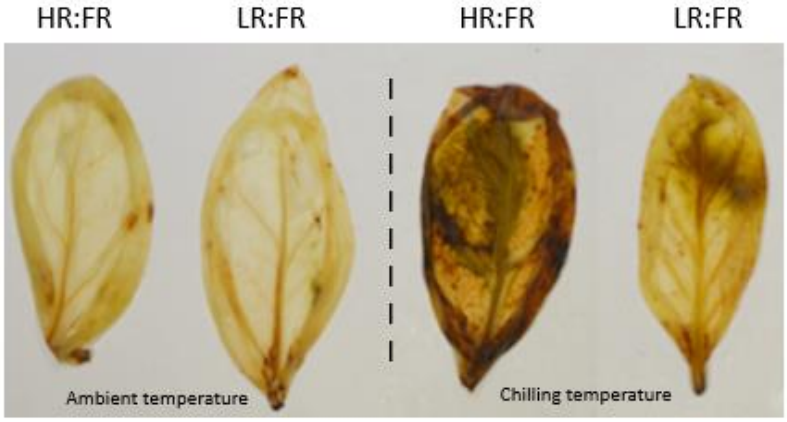


Figure 5.5 DAB assay revealing accumulation of ROS in basil leaves either maintained in HR:FR or treated with periodic LR:FR light (ZT4-8) in a 12 h photoperiod and then exposed to continuous dark chilling conditions (4°C) or continuous dark ambient conditions for 24 h. The tissues are representative of at least n = 15.

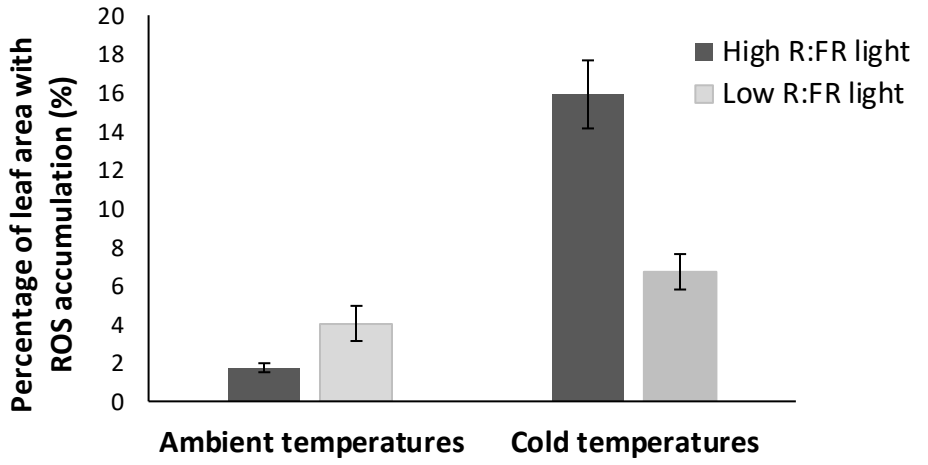


Figure 5.6 % of leaf area with ROS accumulation from basil maintained in HR:FR light or treated with periodic LR:FR light (ZT4-8_ at 12 h photoperiod for four days and then exposed to either continuous dark ambient conditions or continuous dark chilling conditions (4^{o}C) for 24 h. Each bar represents mean ± SE and n = 15.

The Welch ANOVA statistical test showed that there was a statistically significant difference between the various samples (F (3, 68) =31.921, *p = =< .001).* The Games-Howell post hoc test showed that there was significant difference between basil stored at ambient temperatures or chilling temperatures for 24 h, when basil was maintained in HR:FR. However, there was no significant difference between basil stored at ambient temperatures or chilling temperatures for 24 h basil treated with LR:FR light (Figure 5.6) *(p =0.11)*. The accumulation of ROS after treatment of basil with chilling temperatures was significantly lower when basil was pre-treated with LR:FR light than when it was maintained in HR:FR light by 58% *(p = .000)* (Figure 5.5 and 5.6). The results from ROS measurements indicate that LR:FR light is effective in reducing oxidative stress in basil plants during exposure to chilling temperatures and thereby likely to reduce risk of damage to cell and cellular components by ROS.

5.2.3 Effect of LR:FR light on the content of water soluble antioxidants in basil leaves exposed to chilling stress:

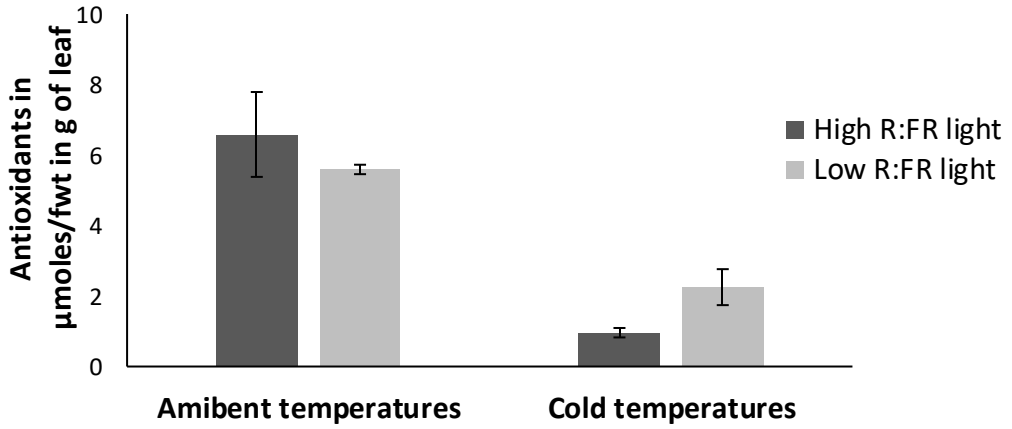


Figure 5.7 Shows the content of water soluble antioxidants (in terms of the equivalent ascorbic acid, µmoles/g fwt of leaf) in leaves of basil either maintained in HR:FR or treated with periodic LR:FR light (ZT4-8) for four days at 12 h photoperiod and then exposed to continuous darkness and ambient conditions or continuous darkness chilling (4^{o}C) conditions for 24 h. Each bar represents mean ± SE and n = 10.

According to a Welch ANOVA test, there was a statistically significant difference across means of basil samples stored at ambient or chilling temperatures and either maintained in HR:FR or pre-treated with LR:FR light (F(3,53)=9.912, *p = < .001*). The Games-Howell post hoc test revealed that there was a significant difference between basil stored at ambient and chilling temperature, regardless of their maintenance in HR:FR *(p = .002)* or treatment with LR:FR light *(p = .027).* Basil declined in its water soluble antioxidants content significantly after treatment at chilling temperatures and was observed to have occurred irrespective of its maintenance in HR:FR or pre-treatment with LR:FR light by 87% and 57%, respectively (Figure 5.7). The difference between basil treated with LR:FR light and subsequent chilling vs ambient temperatures was not found statistically significant *(p = 0.089*) (Figure 5.7). Hence, it appears that LR:FR light had a very important effect on preventing the loss of the content of water soluble antioxidants during chilling. In contrast basil maintained in HR:FR showed a significant decline in antioxidant content (p=0.032).

5.2.4 Effect of LR:FR light on the intactness of PSII indicated by the yield of delayed chlorophyll fluorescence:

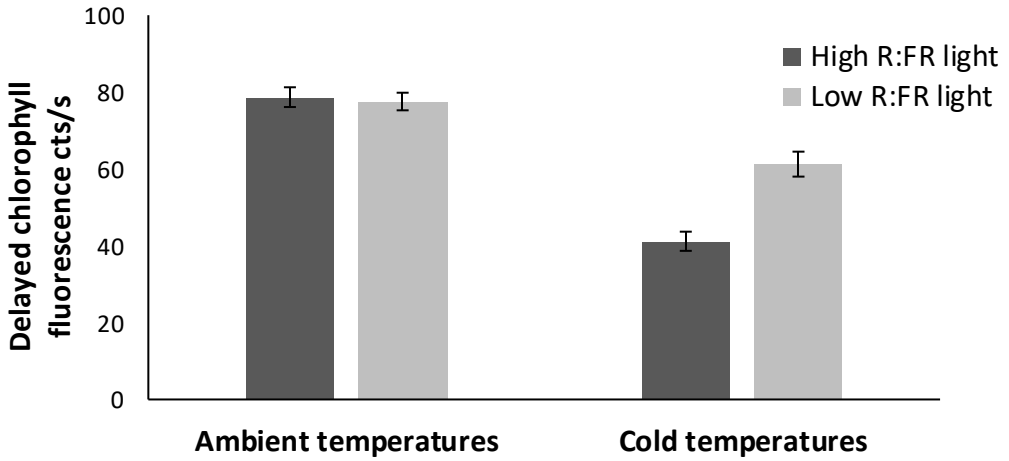


Figure 5.8 Shows the yield of delayed chlorophyll fluorescence in cts/s and thereby represents the content of chlorophyll molecules in basil seedlings either maintained in HR:FR light or exposed to periodic LR:FR light (ZT4-8) at 12 h photoperiod for four days and then exposed to either continuous dark ambient conditions or continuous dark chilling (4°C) conditions for 24 h. Each bar represents mean ± SE and n=15.

The one way ANOVA test revealed that there was a statistically significant difference between means of basil samples stored at ambient or chilling temperature after treatment with either HR:FR or LR:FR light ($F(3,49)=41.429$, $p = .000$). The Tukey HSD post hoc test revealed that there was a significant difference between basil stored at ambient temperatures and chilling temperatures for 24 h when either maintained in control HR:FR light ($p = .000$) or when pre-treated with periodic LR:FR light ($p = .003$) (Figure 5.8). In basil stored at chilling temperatures those pre-treated with LR:FR light yielded a significantly higher yield of delayed chlorophyll fluorescence than basil maintained in HR:FR light by 49% (Tukey HSD post hoc test, $p = .000$) (Figure 5.8). The results of delayed chlorophyll fluorescence suggest that LR:FR light treatment is likely to be effective in reducing loss of chlorophyll content in basil at times of chilling temperature.

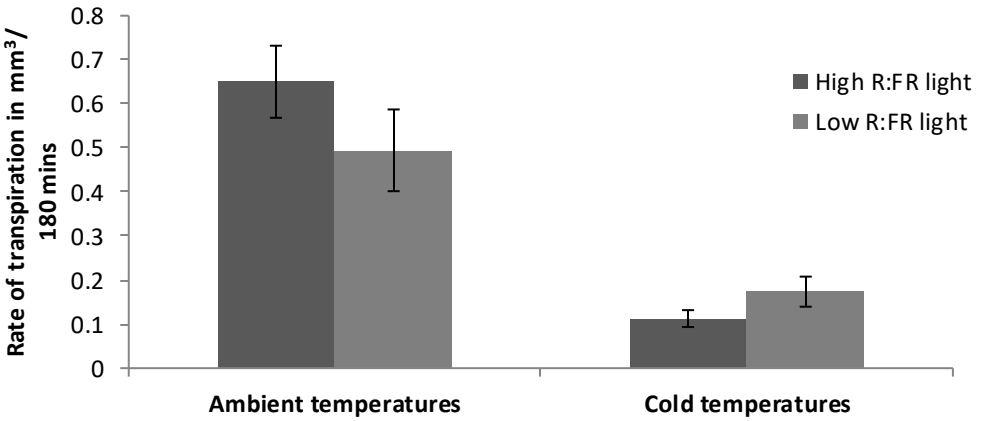
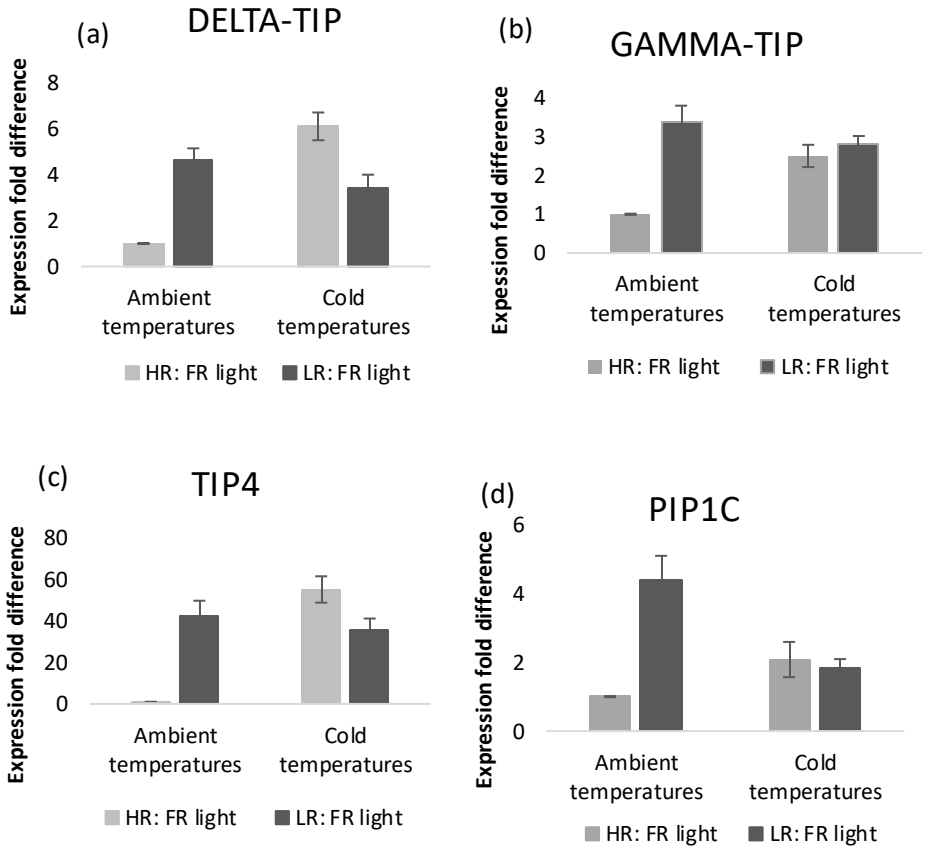


Figure 5.9 Shows rates of water loss (transpiration in mm^3/ 180 mins from basil maintained in HR:FR or treated with periodic LR:FR (ZT4-8) at 12 h photoperiod and subsequently exposed to 24 h of continuous darkness ambient or continuous darkness chilling conditions (4°C) for 24 h. Each bar in the figure represents mean ± is SE and n = 4.

The measurements of transpiration showed that its rate declined in basil after treatment with chilling temperatures irrespective of basil's pre-treatment with HR:FR or LR:FR light (Figure 5.9). One way ANOVA revealed that the difference in the rates of transcription from basil treated at different temperatures and light qualities was statistically significant ($F(3, 12) = 15.693$, $p = .000$). The Post hoc Tukey's test also showed that decline in the rate of transpiration after storage at chilling temperatures in basil either maintained in HR:FR or treated with periodic LR:FR light was statistically significant ($p =< .001$ and $p = .001$, respectively) and was 83% and 11%, respectively (Figure 5.9). However, the difference in the rate of transportation in chilled basil either maintained in HR:FR or pre-treated with LR:FR light was not statistically significant ($p = .908$) suggesting that LR:FR light treatment does not affect basil's transpiration rate in chilling conditions.

5.2.5 Effect of pre-treatment of basil plants with LR:FR light on regulation of aquaporins compared to plants pre-treated with HR:FR light.

The differential gene expression data from the first RNA sequencing experiment showed low gene expression of aquaporins in response to chilling temperature for 24 h under 12 h of photoperiod. Aquaporins play a role in providing tolerance to plants experiencing stress conditions related to water deficit, hence, their regulation by LR:FR light was investigated to determine the impact of the light treatment on their regulation and to establish, if they played a role, in part, in conferring chilling tolerance to chilling sensitive basil (Figure 5.8 a-g).



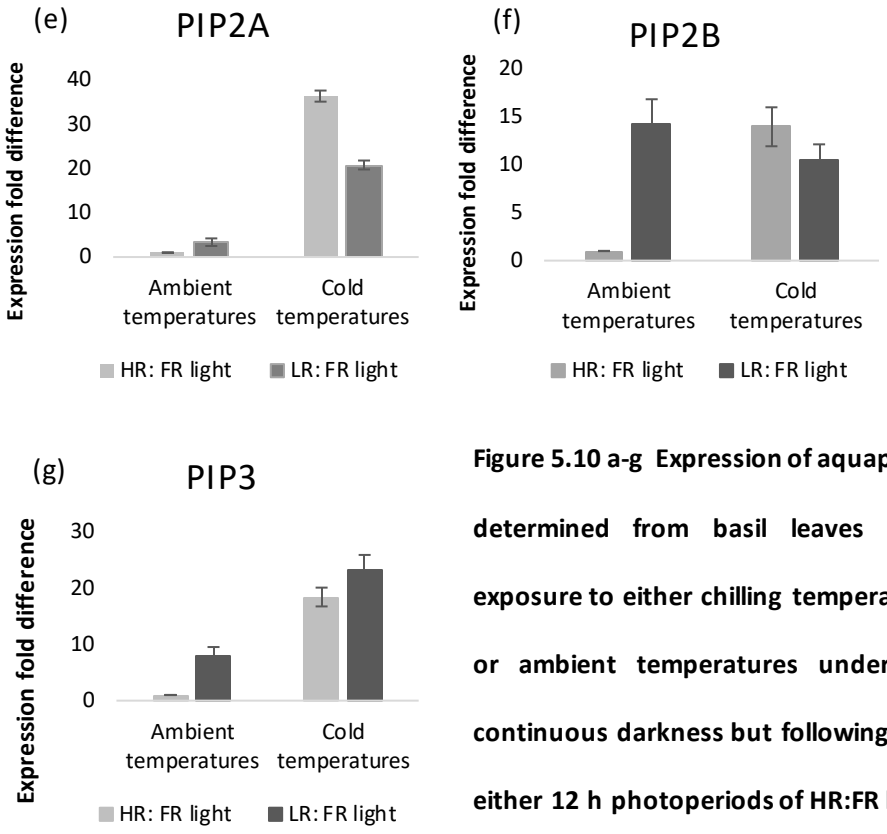


Figure 5.10 a-g Expression of aquaporin genes, determined from basil leaves post their exposure to either chilling temperatures (4°C) or ambient temperatures under 24 h of continuous darkness but following growth in either 12 h photoperiods of HR:FR light or the same conditions with periodic LR:FR treatment (ZT4-8) at ambient conditions for four days. Each bar represents mean of expression fold difference where n = 5 and ± is SE.

The differential gene expression of seven aquaporins (delta-TIP, gamma-TIP, TIP4, PIP1C, PIP2A, PIP2B, and PIP3) was measured by qPCR. The gene expression analysis of all seven aquaporins showed that the basil leaves stored at ambient temperatures for 24 h contained greater gene expression when pre-treated with LR:FR light than when maintained in HR:FR light (Figure 5.10). All seven aquaporins from basil maintained in HR:FR light showed an increase in gene expression after treatment at chilling temperature for 24 h compared to their levels in basil stored at ambient temperatures. In contrast, when basil plants were pre-treated with LR:FR light most genes (delta-TIP, TIP4, PIP1C and PIP2B) showed a decrease and a couple (PIP2A and PIP3) a more minor increase in gene expression after treatment at chilling temperatures for 24 h. Most aquaporin genes from chilled basil plants when pre-treated with LR:FR light showed decreased gene expression compared to their level in basil plants maintained in HR:FR light (Figure 5.10). However,

gamma-TIP genes from chilled basil leaves pre-treated with LR:FR light did not show any difference while PIP3 displayed increased gene expression compared to levels in chilled basil leaves pre-treated with HR:FR light. It appears that the increase in aquaporin gene expression in response to LR:FR treatment mimics that seen in response to cold treatment. It is, therefore, possible that this induction of aquaporins in LR:FR prepares the plant for cold. The fact that expression of aquaporins generally does not increase so much in LR:FR treated plants in response to cold could also support this as, perhaps, this is evidence that these pre-treated plants are not experiencing such a cold shock.

5.2.6 Effect of LR:FR light quality on the relative water content of basil before and after treatment with chilling temperature:

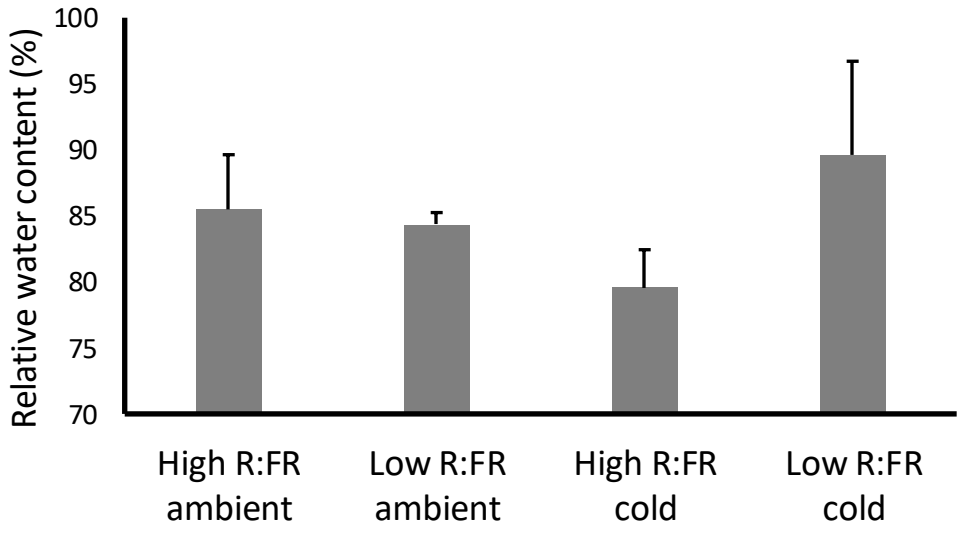


Figure 5.11 Shows difference in relative water content in (%) of leaves from basil maintained in HR:FR light or treated with periodic LR:FR light (ZT4-8) for four days at 12 h photoperiod and subsequently exposed to continuous darkness and ambient conditions or continuous darkness and chilling (4^{o}C) conditions. Each bar represents mean ± SE and n = 20.

The relative water content was measured from basil leaves maintained in HR:FR or treated with LR:FR light and subsequently exposed to 24 h of continuous darkness ambient or

continuous darkness chilling (4°C) conditions (Figure 5.11). According to One way ANOVA a there was no significant change in the relative water content of basil after treatment with chilling conditions when either previously maintained in HR:FR light or pre-treated with LR:FR light ($F(3,76) = 0.896$, $p = .447$). Thus, despite the well documented changes in water relations in other species in response to cold, no such changes were measured in basil. It is, therefore, difficult to extrapolate from this whether the increased expression of aquaporins resulting from LR:FR treatment might have led to any improvement in water relations.

The aim of the Chapter was to investigate the effect of LR:FR light treatment in reducing damage to the cellular components of basil from chilling temperature and thereby induce chilling tolerance in chilling sensitive basil. The physiological assays showed that the membrane permeability, ROS accumulation and loss of chlorophyll content caused by chilling stress was significantly decreased when basil was pre-treated with LR:FR light. Similar findings were obtained in a study by Wang *et al*.[261] in which tomato showed an increase in electrolyte leakage from leaf cells in response to cold when pre-treated with red light but leakage in response to cold decreased following pre-treatment with FR light. The loss of maximum photochemical efficiency of PSII (Fv/Fm) in response to cold was also reduced following pre-treatment of tomato leaves with LR:FR light. According to Wang *et al*[261] mutant studies have shown that LR:FR light treatment of tomato results in activation of phyA and inactivation of phyB where phyA and phyB work antagonistically to regulate the CBF pathway. Active phyA induces ABA signalling that in turn triggers JA signalling leading to increased expression of CBF genes and increased chilling tolerance in tomato plants. In this present study, end of day FR light (EOD-FR) was initially used to try and improve basil's tolerance towards chilling temperatures. However, EOD-FR light produced negative results and was effective in inducing chilling tolerance (see appendix 5.1). There is a possibility that EOD-FR light caused inactivation of phyB but may not have caused activation of phyA and therefore did not cause

Thus, for basil, the observed effects of LR:FR on improving cold tolerance may also be partly due to phyA action as well as phyB action. However, mutants of phyA or phyB showed that the ROS accumulation was positively regulated by phyB more than phyA[341]. Hence, at least for ROS accumulation, it may be much more likely that the reduced ROS accumulation in chilled basil leaves pre-treated with LR:FR light, could be a result of phyB inactivation caused by LR:FR light rather than phyA activation. Reduced accumulation of ROS will result in less damage to membrane, chlorophyll pigments and proteins of the cell during chilling stress[341]**.** In addition, an increased expression of the CBF regulon could provide cryoprotective effects needed for protection against damage by chilling temperatures in sensitive plants. Hence, if the CBF pathway exists in basil then phyB inactivation may not only result in reduced accumulation of ROS but also reduce repression on the expression of CBF genes. PhyA activation by LR:FR on the other hand[341] could possibly cause increased CBF regulon expression resulting in increased chilling tolerance in chilling sensitive basil.

The water regime is disturbed during chilling stress in chilling sensitive plants that leads to water deficit and wilting of leaves[342,343]. Basil plants when stored at chilling temperatures showed a significant decline in the rate of transpiration irrespective of its pre-treatment with either HR:FR light or LR:FR light. Similar findings were obtained by Yu *et al.*[146] where rice varieties when stored at chilling temperatures showed a significant decline in the rate of transpiration. The sensitive variety showed a greater transpiration decline than the tolerant rice variety. Some studies have suggested that the reduced rate of transpiration occurs in response to reduced root water uptake during chilling stress[344,345]. Hence, it was speculated that basil under chilling stress may reduce its rate of transpiration in response to a possible reduced water uptake by the roots. The testing of this speculation of reduced root water uptake would, however, require measurement of root hydraulic conductance to establish if basil plants experienced reduced water uptake by the roots and caused it to show reduced transpiration under chilling conditions. Nevertheless, the results indicate that basil,

when under chilling conditions, could experience water imbalance that could potentially lead to wilting of leaves.

Increased expression of aquaporin genes has been suggested to be associated with increased tolerance of *A. thaliana* to water deficit conditions such as salt or drought[346,347]. In this chapter, the differential expression of seven aquaporins from basil leaves treated with or without LR:FR light and at ambient or chilling temperature was measured. The findings showed that basil under ambient conditions showed greater aquaporin expression in LR:FR treated basil than those that were not. In addition, basil under chilling conditions showed greater aquaporin gene expression in basil not treated with LR:FR light than those treated with LR:FR light. Plants under LR:FR light or vegetative shade elongate to reach above the canopy and receive light rich in red light[205]. Hence, the increase in aquaporin gene expression in LR:FR light treated basil at ambient conditions may have occurred because basil under LR:FR light conditions was probably trying to elongate. Stem elongation requires cell walls relaxation that can occur by increased hydrolytic enzymatic activity or greater intake of water in the vacuole[348]. Hence, increased expression of aquaporins in basil treated with LR:FR light could have occurred to cause greater influx of water and cell expansion. On storage under chilling conditions, basil treated with LR:FR light and possessing greater expression of aquaporins may have experienced less water stress than basil not treated with LR:FR light. Therefore, after storage at chilling temperatures basil treated with LR:FR light may have shown lower aquaporins gene expression than basil not treated with LR:FR light

The height measurement of basil treated with or without LR:FR light did not show a significant difference (appendix 5.1) indicating that the basil plants did not show a shade avoidance behaviour. This was expected as basil plants were treated with LR:FR light for only four days and the duration of the light treatment may be too short for

stem elongation of seedlings to occur. Alternatively, cultivated basil like many commercial crops may have been selected for reduced shade avoidance to optimise its yield at high densities. Nevertheless, increased aquaporin expression in basil plants treated with LR:FR light could be advantageous for basil cells, as it may increase their ability to cope with water deficit induced by chilling stress.

Aquaporin mediate passive transportation of water across the biological membranes, therefore, it is also possible for water movement through increased aquaporin expression to have a detrimental effect on the plant's tolerance to conditions such as salt stress[349]. In grapevine, tolerant to drought but not salt, showed upregulation of PIP21 under salt stress but downregulation under drought stress[350]. Hence, due to the diversity of results obtained on effects of differential regulation of aquaporins on plant responses to drought, the relationship between aquaporins and drought resistance is still somewhat elusive. Analysis of relative water content in this chapter was not able to detect any significant differences in ambient and cold treated basil to provide support to these arguments. The results from studies of the relative water content of basil showed that chilling conditions did not result in a statistically significant loss of water content in basil leaves treated with or without LR:FR light and on their subsequent exposure to chilling conditions (Figure 5.10). This suggests that increased transcript abundance of aquaporins is not exerting any effects on water content of basil tissues. However, although the relative water content results show that the aquaporins do not have any effect on the water balance of basil tissues, the basil treated with LR:FR light does show leaves with greater turgidity following cold than basil treated with HR:FR light, suggesting that increased abundance of aquaporins could possibly have had a positive effect on maintenance of water balance in basil leaves after all. However, the positive effect of aquaporins on maintenance of water balance in basil leaves would require further investigation to be properly elucidated.

For instance, basil can be made to carry mutations such as RNAi lines for any of the aquaporin genes and their roles tested to investigate if low expression of aquaporin genes affect the water content of basil leaves under chilling stress. The duration of basil storage under chilling stress can also be increased to look for any dramatic changes in the speed of basil leaves wilting with aquaporin RNAi lines. Alternatively, basil can be overexpressed with one of the aquaporin gene by creating basil's gain-of-function mutation and its impact can be assessed on basil leaves water content after storage in chilling conditions. Similar approach can be taken to investigate the role of PHYA or PHYB in basil's response to chilling conditions. Basil RNAi lines of either *phyA* or *phyB* can be created and stored under ambient or chilling conditions to investigate whether basil expressing low expression of *PHYA* or *PHY*B can increase or decrease tolerance to cold, respectively.

5.3 Conclusion

This present study shows that LR:FR light is effective in reducing damage to cellular components by chilling temperatures and thereby effective in enhancing chilling tolerance in chilling sensitive basil. LR:FR light pre-treatment led to significantly reduced accumulation of ROS, greater membrane integrity, and prevented loss of chlorophyll and water soluble antioxidants content. The findings obtained from rate of transpiration showed that basil is likely to experience water imbalance during chilling stress and play a role in wilting of basil leaves during chilling stress. LR:FR light pre-treatment also caused basil leaves to contain higher expression of aquaporins that could play a role in increased tolerance to chilling induced water deficit and make the plants more tolerant from damage by cold. Hence, it appears that LR:FR light treatment is able to induce changes that can protect basil cells against damage from ROS and rigidification induced during chilling stress.

The finding of repression on CBF gene expression by active PhyB in the study by Franklin and Whitelam[183] was followed up by Lee and Thomashow[177]. Lee and Thomashow[177] showed that photoperiodic regulation of CBF pathway involved action of phytochromes. Using phyB or pif4 and pif7 double mutants, Lee and Thomashow[177] revealed that phyB physically interacts with pif4 and pif7 to repress the expression of CBF pathway. LR:FR light causes inactivation of phyB and thereby prevent phyB mediated suppression on CBF gene expression. Induction of low temperature tolerance by LR:FR light in the same way as photoperiod regulation of cold tolerance in plants suggests that LR:FR light induced tolerance could be of adaptive value. Before the onset of winter, plants in the autumn conditions experience shortening of days, gradual decrease in temperature and longer twilight (LR:FR) periods. These changes can signal the plant to induce general stress response in plant that may provide protection against damage from sudden exposure to cold[67,183,351].

The LR:FR light quality combined with shortening of days can signal the plant of the forthcoming cold winter conditions and trigger a In addition, CBF pathway is posi tively regulated by the circadian clock during the day, so that the cryoprotective COR polypeptides needed for cold tolerance are synthesized by evening when the temperatures drop even more. Similarly, in this chapter, the LR:FR light was given during the day so that the light treatment synchronizes with circadian clock's positive regulation of CBF pathway. This way, the LR:FR light treatment could stimulate induction of CBF pathway synergistically with the circadian clock and allow maximum activation of the cold regulatory pathway.

Overall, this present study proves that LR:FR light is able to successfully enhance chilling tolerance in basil temporarily. The light regime could be used by Vitacress to reduce basil chilling damage and allow them to treat basil along with other cold tolerant herbs at desired chilling temperatures during transportation. Hence, a

commercial use of LR:FR light regime may possibly make the fresh produce considerable savings by reducing basil wastage and possibly increased sales.

6 PRE-SCREENING OF BASIL'S TRANSCRIPTOMIC PROFILE TREATED WITH OR WITHOUT LR:FR LIGHT BEFORE AND AFTER EXPOSURE TOWARDS CHILLING

6.1 Introduction

Wang *et al*.[261] demonstrated the effectiveness of LR:FR light treatment in inducing chilling tolerance in tomato. Wang *et al*[261] showed the possible involvement of phytohormones in mediating the actions of phytochromes that influence the regulation of CBF pathway in tomato. PHYA and PHYB were found to function antagonistically in their regulation of CBF expression, where PHYB represses but phyA induces the expression of CBF genes[261]. The activation of PHYA by supplementary FR light used in the LR:FR light treatment has been shown previously and acts to moderate the shade avoidance responses that are mediated by removal of phyB Pfr in LR:FR[205]. The fact that phyA is key to cold tolerance in tomato suggests that this is not actually a shade avoidance response but a response to supplementary FR.

Treatment of tomato with LR:FR light during chilling stress of 12 h caused an elevated content of ABA and JA and the plants exhibited greater chilling tolerance than plants treated with HR:FR light[261]. The increase in tomato chilling tolerance was proposed to be a result of active phyA induced ABA and JA signalling[261]. Mutant analysis of phytochromes by Wang *et al* indicated that ABA works upstream to JA where ABA causes activation of COR genes and JA leads to increased expression of CBF genes[259,352,353]. These findings prove that light signalling and phytohormone regulation of plants response to cold stress is integrated and that phytochromes may exert their positive or negative role through functions of phytohormones.

Limited information is available regarding the role of LR:FR light in positively regulating cold tolerance of plants and what is known is based on data from a small group of genes. The information that is available in the literature from the tomato work of Wang *et al*.[261] is also based on changes occurring in plants in response to LR:FR irradiation during their exposure towards chilling temperatures. In this chapter, global

transcriptomic changes occurring in basil treated with or without LR:FR light on the fourth day of their treatment prior to their exposure to chilling stress were investigated. Differences in the transcriptomic profile of basil treated with or without LR:FR light at two different time points ZT6 and ZT10 were determined to propose possible suggestions that may provide information on the role of LR:FR light in inducing chilling tolerance in chilling sensitive basil. The two timepoints were chosen to distinguish between genes showing increased expression only during the LR:FR treatment and those showing longer term changes in expression after the treatment had ended.

6.2 Results and Discussion:

The aim of the chapter was to establish differences in the transcriptomic profile of basil plants treated with or without LR:FR light for a duration of four hours in the middle of the day (ZT4-ZT8). The differences in the transcriptomic profile was thought to provide possible suggestions to basil's (treated with or without LR:FR) differential responses towards chilling conditions. High throughput RNA-sequencing was therefore carried out on four samples where plants were either maintained in HR:FR light or treated with periodic LR:FR light between ZT4-ZT8 for four days. The samples were harvested on the fourth day of the treatment at two different time points ZT6 or ZT10. The sample treated with LR:FR light, where plants were irradiated with additional FR light on top of white light from ZT4 – ZT8 but harvested at ZT10 time point in the absence of FR light will be referred to as ZT10 LR:FR light from here on.

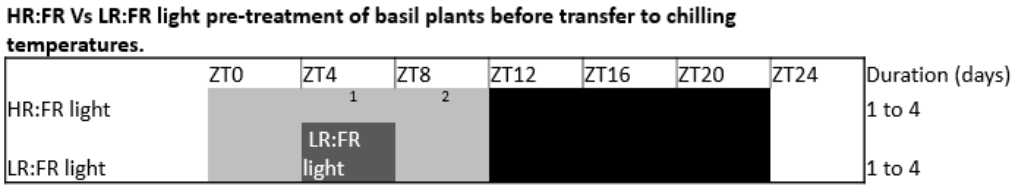


Figure 6.1 The set-up of the RNA sequencing experiment where basil was either maintained in HR:FR light (control) or treated with periodic LR:FR light (ZT4-8) at ambient temperature (22^{0}C) for four days. The LR:FR light treatment involved irradiating basil plants with additional FR light on top of white light for four hours in the middle of the day. The samples were harvested on the fourth day of the LR:FR light treatment where 1 indicates time point ZT6 and 2 represents time point ZT10.

6.2.1 Quality control check of illumina sequencing raw data:

RNA sequencing of basil samples was carried out by Dr Sandra Smieszek (Case Western University, USA). The sequence file of the four samples was assessed for their quality using the FastQC software. RNA sequencing of four basil samples including HR:FR light

at ZT6, LR:FR light at ZT6, HR:FR light at ZT10 and LR:FR light at ZT10 produced a total number of 40 million, 36 million, 49 million and 43 million reads, respectively. The total length of each read each was 51 bases long after removal of indexing primers. for the library of reads for all four samples. The assessment of the quality of the base calls was carried out using the Sanger Illumina 1.9 encoding method. On average the quality (Phred) scores at which the bases were called were found to fall into the very good calls (green >28) region and produced a mean score of 38. A Few bases whose score fell into the region of poor quality (red) with a score of 15 or below were trimmed from the ends of specific sequences in the data. The quality assessment per sequence overall after trimming was found to show a single tight distribution with a quality Phred score of 38, demonstrating that the library of reads did not contain any group of sequences with bases of poor quality call score. This, in turn, indicated the reads to be of good quality overall. The libraries were also not found to contain any adapters across all reads (these having been already removed) or any N calls across all bases, demonstrating that the libraries of all four samples were of good quality.

6.2.2 Comparisons of basil treated with four different conditions (HR:FR light at ZT6, LR:FR light at ZT6, HR:FR light at ZT10 and finally LR:FR light at ZT10) based on their gene expression data:

In order to gain a clear understanding of the differences that exist between basil treated with or without LR:FR light and at the two different time points, multiple comparisons of the four samples were carried out using pattern fitting software and the clustering tool of mapman. Multiple comparisons would allow analysis of changes in both global and individual gene expression across all four samples collectively and is more informative in providing information regarding the role of LR:FR light in

positive regulation of cold tolerance than pairwise comparison where gene expression analysis can be carried out in only two samples at a time.

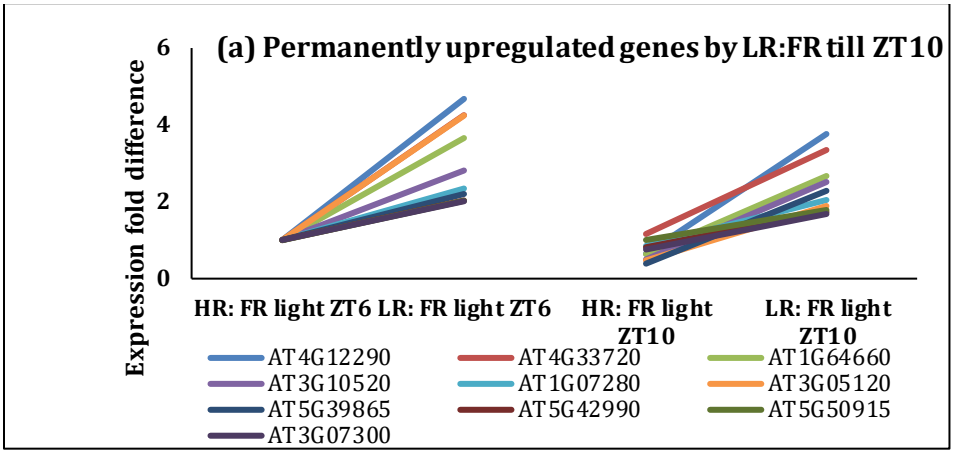
From the 7651 unigenes identified and found to share significantly high homology with transcripts of *A. thaliana*, 2568 unigenes were identified as interesting based on their conformation to one of six unique gene patterns. Among these six patterns, four of them were chosen in advance as being patterns of interest. The correlation of the expression patterns of individual genes to these chosen patterns (correlation cut-off $p<0.05$) was assessed using pattern fitting software[205], a method also called biased clustering. The remaining two patterns were established using the clustering tool of Mapman[206] using non-biased K-means clustering as being additional patterns shown by significantly large groups of genes.

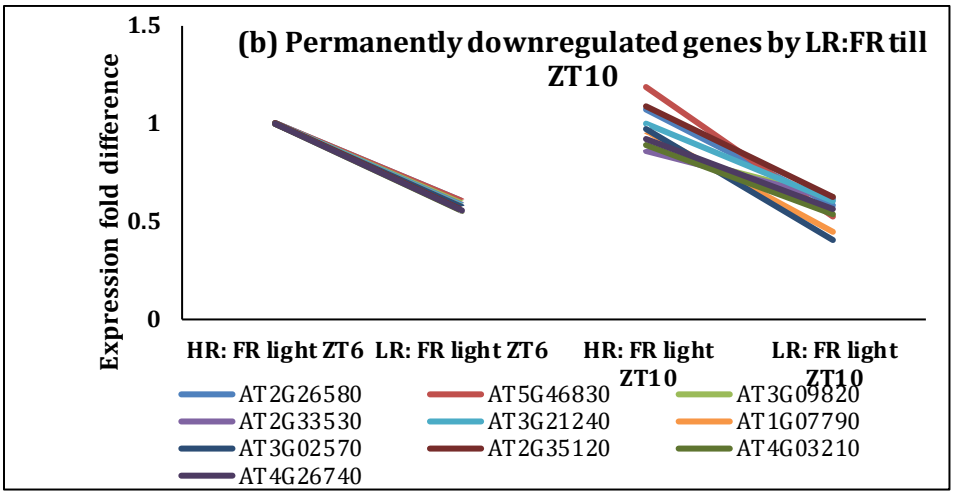
The first chosen pattern of interest identified 37 genes that showed upregulation in response to LR:FR light at ZT6 and maintained their regulation up to time point ZT10. These genes were termed "permanently" upregulated in response to irradiation by LR:FR light from ZT4-ZT8 (Figure 6.2a). The second pre-determined pattern identified 37 genes that were downregulated in response to LR:FR light at ZT6 and maintained their downregulation up until ZT10. Hence, these genes were termed "permanently" downregulated in response to LR:FR light irradiation from ZT4 - ZT8 (Figure 6.2b). The third pattern identified 1573 genes that showed upregulation to LR:FR light at ZT6 but returned to their original expression level by ZT10 and were, therefore, referred to as genes that showed temporary upregulation to irradiation by LR:FR light from ZT4-ZT8 (Figure 6.2c) and finally the fourth pattern identified 100 genes that showed temporary downregulation in response to irradiation of LR:FR light at ZT6 but returned to their original expressional level by ZT10 (Figure 6.2d). The two clusters or patterns established through mapman non-biased clustering include 253 genes that showed upregulation by LR:FR light at both ZT6 and at ZT10 but which showed a significant

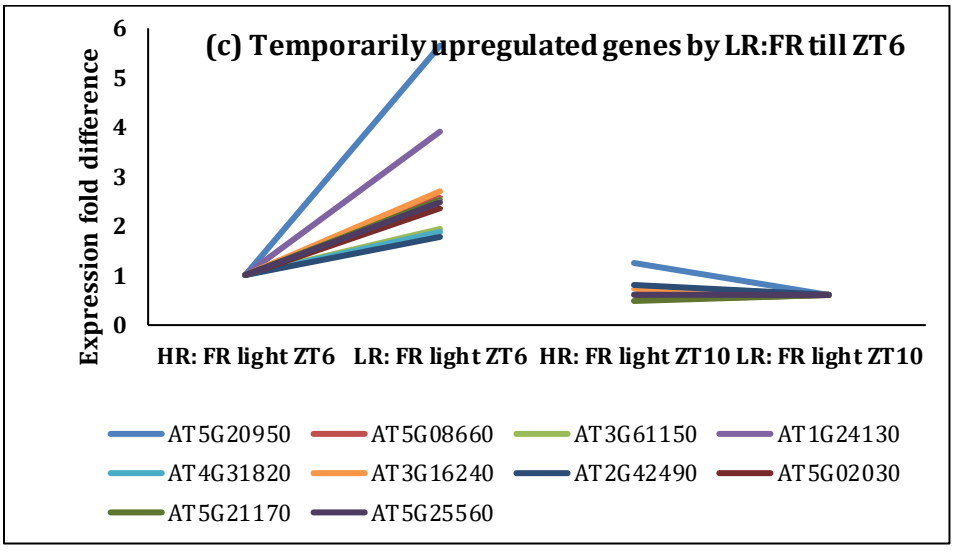
drop in basal expression in control plants between ZT6 and at ZT10. The drop in basal expression was sufficient to cause them not to correlate to pattern 1. This represents a significant number of genes suggesting that this is a common feature of LR:FR light responsive genes in basil (Figure 6.2e). A second Mapman cluster identified 26 genes that showed upregulation only by LR:FR light at ZT10 (Figure 6.2f).

The vast difference in the number of genes found in each cluster represents the responsiveness of basil's transcriptome to LR:FR light (Figure 6.2 a-d). For instance, from the 1573 genes that were upregulated in the presence of LR:FR light, only 137 genes remained upregulated after removal of the LR:FR light. The return of the large number of genes back to their basal expression, once the LR:FR light was removed, indicates that LR:FR light was continuously being monitored by basil's transcriptome apparatus.

The number of genes upregulated were higher than the number of genes downregulated in all of the four genes clusters including permanent or temporary among the up or downregulated genes. This observation indicates that LR:FR light is likely to have had a stimulatory effect on basil's transcriptome. The LR:FR light treated basil plants with greater induced genes expression may possibly contain more defence mechanisms that may contribute to LR:FR light treated increased chilling tolerant behavior. Hence, the patterns described above are of interest to investigate further and explore the biolgical processes they affected that might provide an insight of the way LR:FR light affected basil plants.


(a) Permanently upregulated genes by LR:FR till ZT10
Expression fold difference
6
4
2
0
HR: FR light ZT6
LR: FR light ZT6
HR: FR light ZT10
LR: FR light ZT10
AT4G12290
AT4G33720
AT1G64660
AT3G10520
AT1G07280
AT3G05120
AT5G39865
AT5G42990
AT5G50915
AT3G07300


(b) Permanently downregulated genes by LR:FR till ZT10
Expression fold difference
1.5
1
0.5
0
HR: FR light ZT6
LR: FR light ZT6
HR: FR light ZT10
LR: FR light ZT10
AT2G26580
AT5G46830
AT3G09820
AT2G33530
AT3G21240
AT1G07790
AT3G02570
AT2G35120
AT4G03210
AT4G26740


(c) Temporarily upregulated genes by LR:FR till ZT6
Expression fold difference
6
5
4
3
2
1
0
HR: FR light ZT6
LR: FR light ZT6
HR: FR light ZT10
LR: FR light ZT10
AT5G20950
AT5G08660
AT3G61150
AT1G24130
AT4G31820
AT3G16240
AT2G42490
AT5G02030
AT5G21170
AT5G25560

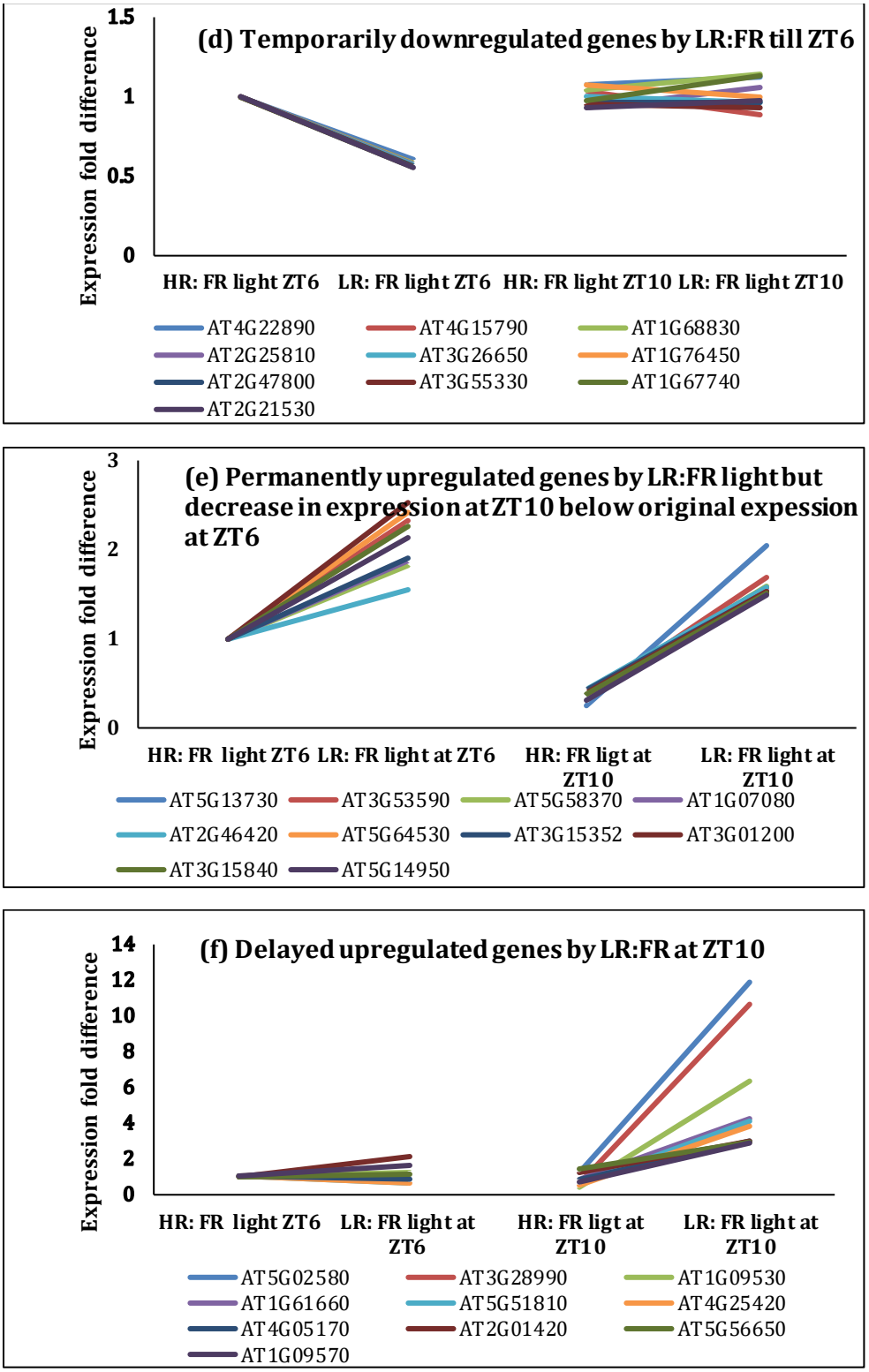


Figure 6.2 a-f patterns of gene expression of basil exposed to four different conditions that include HR:FR light at ZT6, LR:FR light at ZT6, HR:FR light at ZT10 and LR:FR light at ZT10 are demonstrated. In each pattern only top ten genes of basil sharing high homology with that of *A. thaliana's* transcript TAIR ID were displayed. The TAIR ID of the remaining genes that are involved in each unique pattern can be

found in appendix 6.1-6.6. Patterns a - d show genes that are permanently upregulated, permanently downregulated, temporary upregulated and temporary downregulated in response to LR:FR light irradiation from ZT4-ZT8, respectively (a-d). The total number of genes fitting to patterns 6.2 a-d are 37, 37, 1573, and 100, respectively. Pattern e shows genes which are permanently upregulated by LR:FR treatment but which show a drop is basal expression between ZT6 and 10 in untreated plants. (e). Pattern f shows genes which exhibit upregulation only in LR:FR at ZT10 (f). For patterns 6.2 e – f, n = 253 and 26.

6.2.3 Biological processes enriched among genes that showed permanent upregulation to LR:FR light irradiation from ZT4-ZT8:

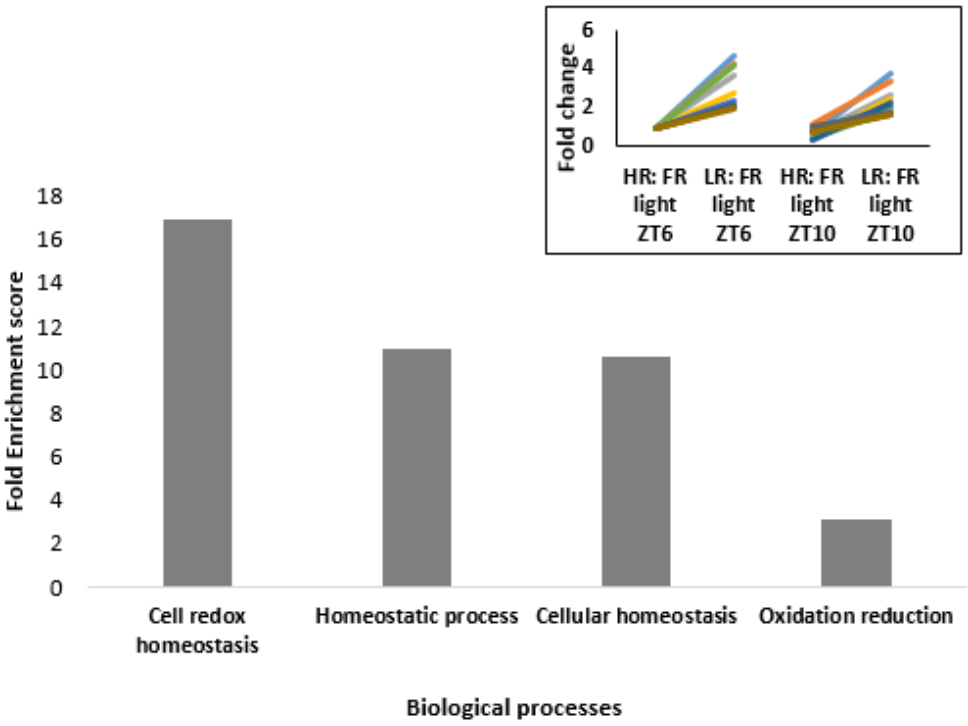


Figure 6.3 Biological processes found significantly enriched (p<0.05 based on Fisher's Exact p value by EASE) among permanently upregulated genes in response to LR:FR light irradiation from ZT4 – 8. The icon of the permanently upregulated genes cluster was added to show the pattern of genes being discussed.

Genes that belong to the pattern of permanent upregulation to LR:FR light without a change in expression in untreated plants were found to be significantly enriched with biological processes such as cell redox homeostasis, homeostatic process, cellular homeostasis and oxidation-reduction (Figure 6.3) (For genes annotated to each biological process see appendix 6.7). These biological processes included the glutaredoxin-C1, monothiol glutaredoxin -S2, peroxiredoxin-2F and cytochrome P450 90C1 genes whose proteins play roles in detoxification of ROS content in leaves as well as in limiting the further generation of oxidative stress in the cell[68,354,355]. The upregulation of genes coding for proteins with properties of detoxification of ROS in

LR:FR light treated plants was surprising, as LR:FR light causes removal of phyB that is involved in positive regulation of ROS[341]. Therefore, LR:FR light treatment was expected to cause lower accumulation of ROS than basil maintained in HR:FR light. Hence, it may be possible that the increased expression of enzymes coding for proteins with ROS scavenging properties are likely to be induced by mechanisms other than increased ROS content of the cell. Regardless of the cause that may have led to increased expression of ROS scavenging proteins, the possibly increased abundance of antioxidative enzymes in LR:FR light treated basil is likely to provide them with greater protection against oxidative stress during chilling stress [356]. Enriched cellular location of permanently upregulated genes in response to LR:FR light were chloroplast envelope, chloroplast part and plastid part suggesting that the chloroplast may be an important site of action in mediating more permanent LR:FR light responses in the cell.

6.2.4 Biological processes enriched among genes that showed temporary upregulation to LR:FR light irradiation from ZT4-ZT8:

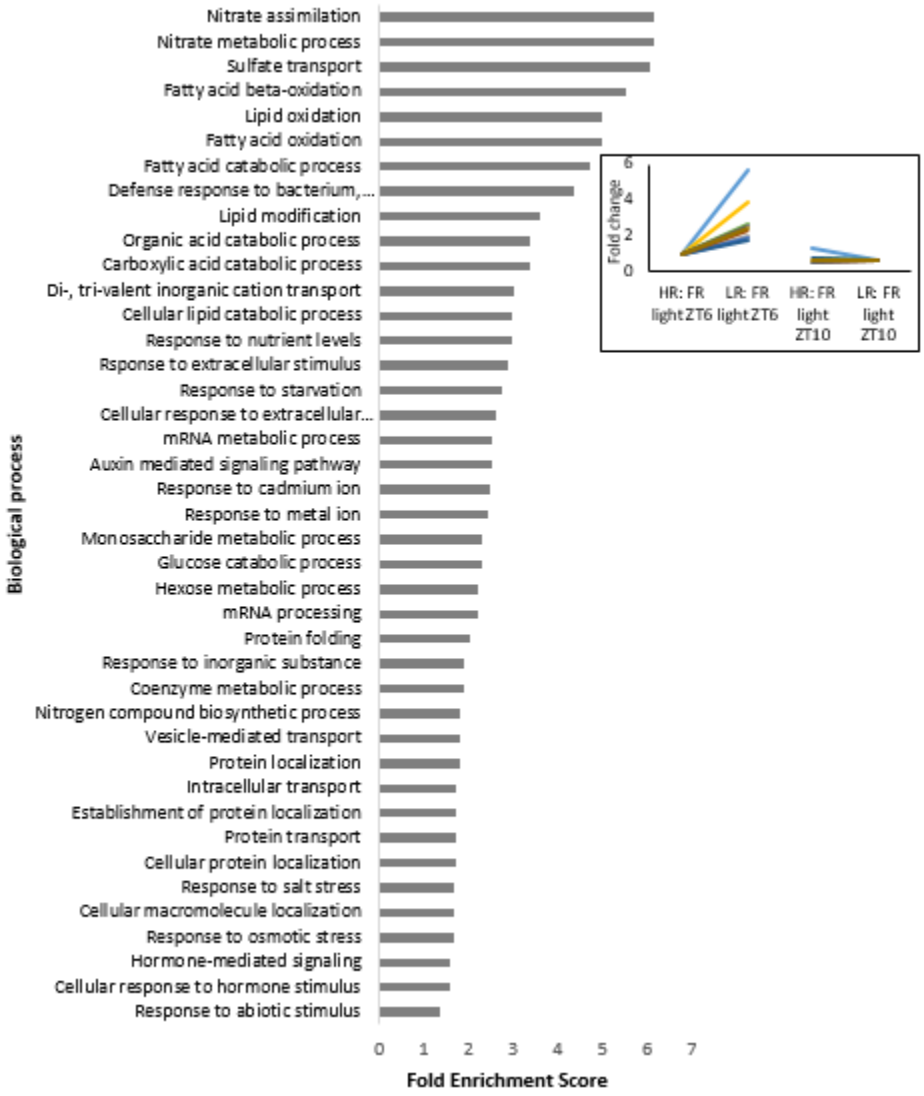


Figure 6.4 Enriched biological processes that (p<0.05 based on Fisher's Exact p value by EASE) among temporarily upregulated genes in response to LR:FR light irradiation from ZT4 – 8. The temporary upregulated genes cluster is also shown for visualisation of genes behaviour. The icon of pattern containing genes showing temporary upregulation is also shown to demonstrate the behaviour of genes being discussed.

Genes belonging to the pattern of temporary upregulation in response to LR:FR light at ZT6 and did not show any change in untreated plants included 160 significantly enriched biological processes (appendix 6.8). From 160 biological process, the most

significantly enriched 40 biological processes (25% of 160) are shown (Figure 6.4). The biological processes discussed here include, nitrate assimilation, nitrate metabolic process, sulfate transport, Lipid oxidation, Fatty acid oxidation, lipid modification, fatty acid catabolic process, organic acid catabolic acid, defense response to bacterium, process response to extracellular stimulus, response to cadmium ion, response to metal ion, response to inorganic substance, nitrogen compound biosynthetic process, response to salt stress, response to osmotic stress and hormone-meditated signalling (Figure 6.4).

Nitrogen is one of the most important constituents of the cell and its assimilation is vital for the growth and development of the plant[357]. Once taken up by the root in the form of nitrate, nitrogen is reduced to ammonium ion by nitrate reductase and incorporated into amino acids[357]. According to Cyr *et al.*[358] chilling resistance is associated with the increased content of saccharides, nitrate, amino acids and total protein content in the cell. In a study by Mostaffa and Hassan[151] *Azolla caroliniana* plants chilled for 7 days and then transferred into either nitrogen free Hoagland solution or Hoagland solution containing KNO_3. Non chilled *Azolla caroliniana* plants consisted of greater protein content than chilled plants but the protein content was higher in the chilled plants in the presence of nitrogen than in the absence[151]. Thus, the presence of nitrogen source and increased nitrogen assimilation was proposed to alleviate chilling injury symptoms in plants.

In this present study, the biological process nitrate assimilation is annotated with genes such as SNF1 kinase homolog (KIN10), KIN11 and KING1. KIN10 and KING1 is involved in assimilation of nitrogen by phosphorylating nitrate reductase[359]. KIN11 is involved in promoting plants tolerance to drought conditions by causing induction of stress responsive genes such as RD29, COR15A, CBF3 and ABA and influence expression of stress inducible genes[360–362]. These genes are also known to provide tolerance to

cells from damage by low temperature[64,261,363,364]. Hence, temporary upregulation of genes annotated to nitrate assimilation may be beneficial and increase basil's adaptability to cold conditions.

Sulfate transport included genes such as sulfate transporter 1.1, sulfate transporter 1.2, sulfate transporter 1.3, sulfate transporter 2.1, and sulfate transporter 2.2. Increased expression of sulfate transporters can increase the distribution of sulfate around the cell and confer tolerance of plants towards drought and cold stress. Sulfate is used as a precursor for the synthesis of ABA and glutathione[365] that can result in increased expression of CBF target genes[261].

Lipid oxidation, Fatty acid oxidation, lipid modification, fatty acid catabolic process and organic acid catabolic acid process were annotated to genes including ABC transporter COMATOSE (CTS), 3-ketoacyl-CoA thiolase, acyl-coA-oxidase 1, acyl-coA-oxidase 3, acyl-coA-oxidase 4 and acyl-coA-oxidase 5 where CTS, acyl co A oxidase 1 - 5 were suggested to be involved in the synthesis of JA[366–368]. JA is needed for CBF gene expression and therefore upregulation of acyl oxidase 1 and 5 may contribute to basil plants adaptation to cold temperatures.

Defence response to bacterium included gene such as quinone reductase, HSP70, ethylene insensitive 3 family protein (EIN3) and calcium ATPase (ACA4 and ACA11) where quinone reductase , HSP70 and EIN3 play roles in protection of cells from adverse effects of oxidative stress[369–371]. EIN3 removes ROS by inducing expression of peroxidase[371] while quinone reductase scavenges ROS directly[372] and HSP70 was involved in removing oxidised protein by inducing their proteasomal degradation[370]. Ca^{2+} ATPase are responsible for maintaining normal cellular Ca^{2+} concentration[373]. Ca^{2+} ions are increased in plant cells under stress conditions including salt, drought, and cold[61]. However, accumulation of Ca^{2+} ions have also been suggested to be associated with activation of programmed cell death pathway in plant cells[374]. Ca^{2+} ATPase

channels are located on the plasma membrane, endoplasmic reticulum and tonoplast where they can pump Ca^{2+} ions into the apoplast against its electrochemical gradient[61]. Study by Boursiac *et al.* using knock out mutants of ACA4 and ACA11 showed that their presence can supress the salicylic acid dependent programmed cell pathway. Thus, temporary upregulation of genes involved in ROS and Ca^{2+} ions dismissal from cells may be beneficial for basil cells and make them better able to cope with damaging conditions possibly imposed by chilling stress.

Response to extracellular stimulus, response to cadmium ion, response to metal ion and response to inorganic substances included the genes aconitate hydratase 2, aconitate hydratase 3, formate dehydrogenase, glutamate dehydrogenase 1, glutamate dehydrogenase 2, glycine-rich RNA binding protein 7, heat shock cognate 70 kDa protein 1, catalase 2, catalase-3 and peroxidase 42. All of these genes code for proteins with roles linked to redox homeostasis and preventing cell from adverse effects of oxidative stress[370,375–379]. Previous data in this thesis showed that cold responses in basil involved considerable changes in redox status and redox associated gene expression. Therefore, these genes that are also induced by LR:FR light are potential genes of interest in LR:FR induced chilling tolerance.

Metal ion stress generally results in increased toxicity of ROS in plants[380] and hence the genes annotated by the metal ion stress biological process consist of functions in detoxification of ROS. Metals ions are taken up from the uptake of water in the soil, therefore, the occurrence of metal ion stress in basil is probably not dependent on the light quality used. However, the response of the plant to a possible generation of metal ion stress may vary from basil plants treated with different light conditions. Basil treated with LR:FR light showed higher expression of genes (mentioned above) with ROS scavenging properties than basil plants maintained in HR:FR light, therefore LR:FR light treated basil appears to have built up mechanisms that may protect the plant

from adverse effects of metal ion stress and cold stress. Most intriguingly, the majority of these temporarily LR:FR-induced genes in these "response to inorganic substance" categories are the same as those highlighted as part of the "response to cold" cluster of genes that were induced in response to cold in the previous gene expression analysis in cold treated basil. This also highlights these genes as particularly interesting for further analysis in the process of exploring the mechanism of the cryoprotective effect of LR:FR treatment.

The genes annotated to the response to salt stress biological responses include ABF, MAPK, aldehyde dehydrogenase and osmotin. Plant cells under LR:FR light elongate to reach above vegetative canopy and absorb light rich in red light[381]. The elongation of stems under LR:FR light is mediated by expansion of cells through increased uptake of water. The increased water uptake in cells may reduce dehydration of cells during chilling, salt or drought stress. Hence, some of the molecular changes occurring in plants in response to LR:FR light may therefore overlap with response to salt and drought stress. It was therefore not surprising to find the biological process of response to salt stress as enriched among LR:FR light temporary upregulated genes. LR:FR light in this present study has caused increased expression of ABF gene that is involved in inducing expression of ABA responsive genes[382]. ABA signalling in other studies has been suggested to increase in plants under LR:FR light treatment[261,383]. The increase in ABA signalling is also observed in plants response to salt and drought stress[384,385] where ABA prevents the uptake of salt by the cells by increasing the cells uptake to $Ca^{2+,}$ K^{+}, sugars and other solutes[386]. ABA also causes increased expression of MAPK[387] and was found temporarily upregulated by LR:FR light treatment in this present study. MAPK under salt conditions functions to prevent loss of water by limiting the number of stomatal opening in plant cells. Salt stress in plants also leads to ROS accumulation and hence temporary upregulated genes by LR:FR light such as aldehyde dehydrogenase with roles in scavenging of ROS was also found annotated to

salt stress biological process. Genes mentioned above with roles such as reducing loss of water from cells, accumulation of solutes and in scavenging of ROS may also provide protection to basil cells from adverse effects of chilling stress.

Finally, the GO term hormone mediated signalling included auxin response factors, ethylene-responsive transcription factors, auxin signalling F-Box 3, Transport inhibitor response 1, serine/threonine protein kinase (CTR1) and serine-threonine protein kinase (SRK2I). Here, it appears that auxin signalling was likely to have increased as well as genes responsive to increased ethylene levels in the cell. At ambient temperatures, induced auxin signalling causes the growth of stems - a commonly observed shade avoidance response of plants under LR: R light irradiation [388]. It is most likely that such hormone associated genes are simply involved in regulating elongation growth responses associated with shade avoidance. This, therefore, makes them low priority genes in terms of our interest in induction of cold tolerance. Non-the-less, the fact that such patterns of expression in response to shade show strong similarity to those observed in *A. thaliana*[205] provides some degree of confirmation that changes observed here do represent genuine LR:FR induced gene expression changes. ABA and ethylene, on the other hand, are hormones that can be induced in plants in response to abiotic stresses such as drought, wounding and cold[389,390]. The increased content of ABA and ethylene can induce expression of genes such as that of CBF regulon and ERF domain containing ethylene transcription factors that in turn can cause induced expression of stress-responsive genes [389]. This increases plants ability of defense against the abiotic stress. Thus, ABA and ethylene associated genes may be of further interest.

The temporary upregulated genes annotated to enriched biological processes show that genes involved in redox homeostasis of the cell is of importance. Biological processes annotated with genes linked with redox homeostasis were also found

enriched in the cold treated basil (first RNA sequencing experiment). Thus, upregulation of similar genes (catalase 2, catalase 3, aconitate hydratase 2, aconitate hydratase 3, glycine rich RNA binding protein and HSP70) in cold treated basil as well as those treated LR:FR light at ambient temperatures suggests for those genes to play important role in basil's tolerance to chilling temperatures. Many of the LR:FR light upregulated genes annotated to enriched biological processes were involved in the synthesis of ABA or JA, indicating their role to also be of importance in basil's development of chilling tolerance. The upregulation of genes involved in synthesis of ABA and JA among LR:FR light treated basil plants was expected and may possibly be a result of LR:FR light mediated phyA activation[261]. The enriched GO terms of temporary upregulated genes in response to LR:FR light provide an insight into the possible roles of redox homeostasis, ABA and JA induction in basil's development of chilling tolerance. Thus, upregulation of these genes in LR:FR light makes them more tolerant to chilling temperatures than basil maintained in HR:FR light.

The genes, however, were upregulated temporarily but their gene products would be present at a higher abundance than in plants maintained in HR:FR light. However, it is impossible to predict the duration of the gene products effect on basil cells with any certainty and would, therefore, require an accompanying proteomic analysis in order to properly address this.

6.2.5 Biological processes enriched among genes that showed permanent downregulation to LR:FR light irradiation from ZT4-ZT8:

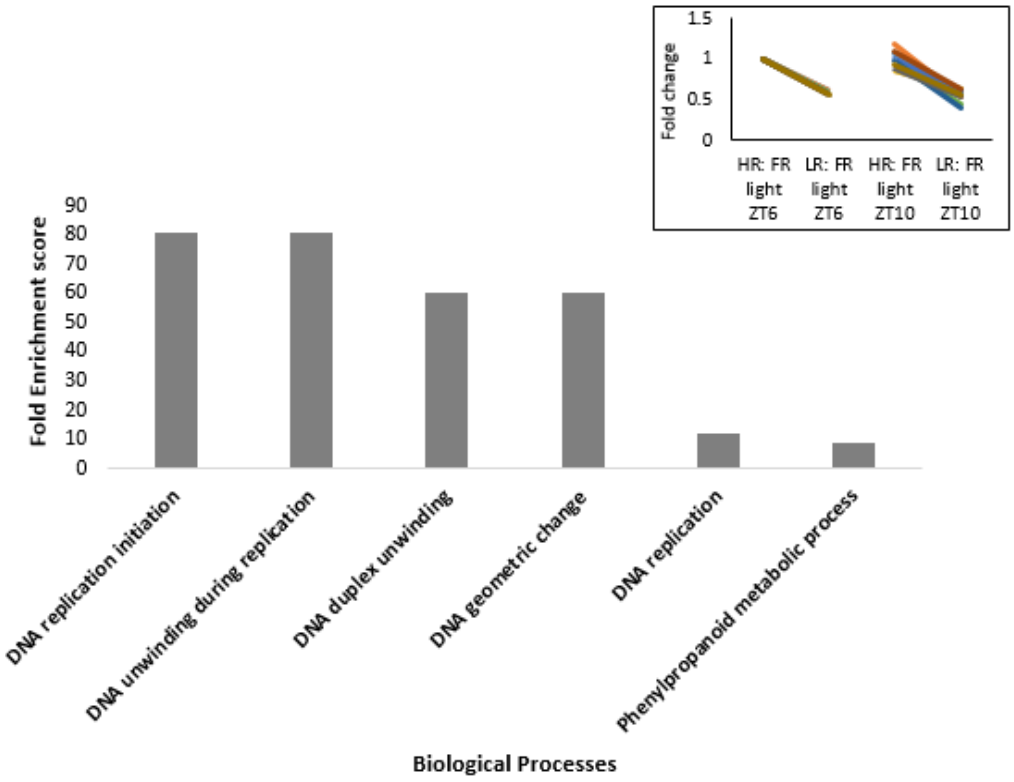


Figure 6.5 Enriched biological processes among permanently downregulated genes ($p<0.05$ based on Fisher's Exact p value by EASE) in response to LR:FR light from ZT4 – 8. The icon of pattern containing genes showing permanent upregulation is also shown to demonstrate the behaviour of genes being discussed.

Six biological processes including DNA replication initiation, DNA unwinding during replication, DNA duplex unwinding DNA geometric change, DNA replication and phenylpropanoid metabolic process were found significantly enriched among permanently downregulated genes in response to LR:FR irradiation from ZT4-ZT8 (Figure 6.5) (For genes annotated to each biological process see appendix 6.9) . AT2G16440 and AT5G44635 were found commonly annotated by DNA replication

initiation, DNA unwinding during replication, DNA duplex unwinding and DNA geometric change. It is likely that a downregulation of DNA replication biological processes by LR:FR light would be a natural consequence of a cessation of cell division growth associated with stress responses in general.

6.2.6 Biological processes enriched among genes that showed temporary downregulation to LR:FR light irradiation from ZT4-ZT8:

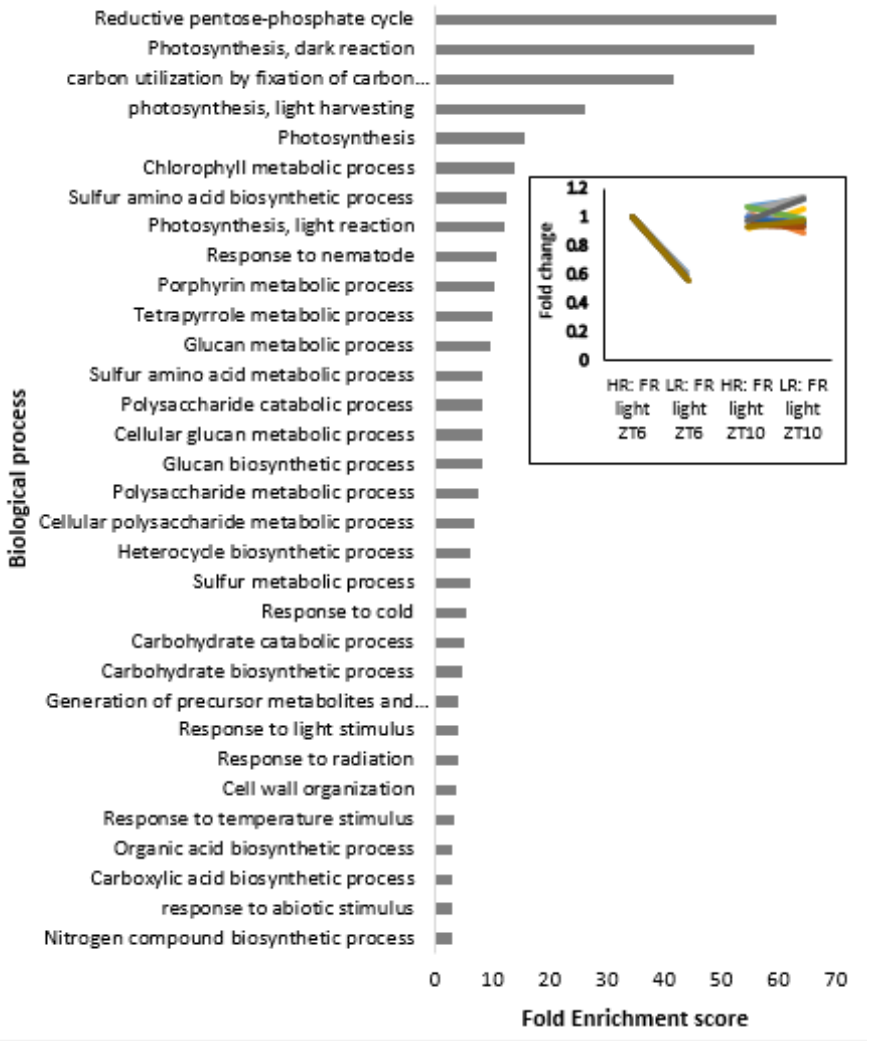


Figure 6.6 Shows biological processes that have been found significantly enriched ($p<0.05$ based on Fisher's Exact p value by EASE) among temporarily downregulated genes in response to irradiation of LR:FR light from ZT4 – ZT8. The icon of pattern containing genes showing temporary downregulation is also shown to demonstrate the behaviour of genes being discussed.

32 significantly enriched biological processes were identified from list of basil genes that showed temporary downregulation to LR:FR light irradiation from ZT4-ZT8 (Figure 6.6 and details of the genes annotated to the biological process are provided in appendix 6.10).

The biological processes discussed here include reductive pentose-phosphate cycle, photosynthesis, and carbon utilisation by fixation of carbon dioxide, photosynthesis light reactions, photosynthesis dark reactions, carbohydrate biosynthetic process, chlorophyll metabolic process, sulphur amino acid metabolic process, photosynthesis light reaction, carbohydrate catabolic process and response to cold

Reductive pentose-phosphate cycle, photosynthesis, carbon utilisation by fixation of carbon dioxide, photosynthesis light reactions, photosynthesis dark reactions, carbohydrate biosynthetic process, carbohydrate catabolic process (Figure 6.6). These processes include the genes, photosystem II 22 kDa protein, photosystem II 5 kDa protein, Photosystem II core complex psbY, and Psbp like protein 1. The genes mentioned are all components of PSII complex and involved in protection of PSII from either photooxidative damage, repairing photo-damaged PSII reaction centre, photoinhibition or in coping with reduced Ca^{2+} ion concentration in the cell[391–394]. PSII 22 kDa protein is involved in non-photochemical quenching to maintain the balance between light energy absorbed and used by photosynthesis and thereby limits the generation of oxidizing molecules[391]. *Chlamydomonas reinhardtii* deficient in PsbT showed reduced PSII activity under strong light and reduced in its recovery considerably after partial photo-inactivation[392]. Hence, PsbT was suggested to be important for photo-damaged recovery of PSII reaction centre. Lastly PsbY and PsbP are core components of PSII complex where PSII in *A. thaliana* deficient in PsbY showed

more susceptibility to photoinhibition[393] and PsbP was found important for Ca^{2+} and Cl^- retention in the cell[394].

Genes annotated to photosynthesis light harvesting process and porphyrin metabolic process include Chlorophyll a- b family binding protein (LIL3.2) and light harvesting complex (LHCA3). LIL3.2 gene is LHC like protein, part of light harvesting complex for PSII and involved in chlorophyll biosynthesis[395]. Transgenic plants with reduced amounts of LIL3.2 resulted in impaired growth, reduced chlorophyll and carotenoid content compared to wild type plants[395]. An increased expression of LIL3.2 was also observed in *A. thaliana* under high light stress, hence, LIL3.2 was suggested to be involved in stabilisation of LHC of PSII under stress conditions[396]. The light harvesting complex (LHCA3) functions as a light receptor that absorbs light and transfers excitation energy to the reaction centre of photosystem it is most closely associated to. Hence, downregulation of LHCA3 and LIL3.2 may possibly affect photosynthetic activity of basil cells and have reduced protection for PSII against photooxidativae damage.

The downregulation of genes annotated to photosynthesis and photosynthesis light harvesting processes show that pants treated with LR:FR light are probably more susceptible to photo damage under chilling stress than plants pre-treated with HR:FR light. However, in this present study the basil plants were stored under continuous darkness when subjected to chilling temperature. Hence, although basil plants are probably disadvantaged with reduced protection towards PSII but may possibly not suffer much due to the conditions the basil plants are given during chilling stress.

The downregulation of genes involved in Calvin cycle , response to cold, photosynthesis dark reaction and carbohydrate biosynthetic processes such as glyceraldealdehye-3-phosphate dehydrogenase A and glyceraldeal dehye-3-phosphate dehydrogenase B, phosphoribulokinase and the sedoheptulose-1,7-bisphosphatase is

speculated in this present study to have possibly occurred in consequence to the reduced yield of light reactions. Overall, this downregulation of photosynthesis-associated gene expression seems unlikely to have caused a large effect on plants ability to cold acclimate.

Genes annotated to chlorophyll biosynthesis included Mg-protoporhyrin IX monomethyl ester (MPE), CDR1, CHLH. Aerobic Mg-protoporhyrin IX monomethyl ester (MPE) is a key enzymes involved in chlorophyll biosynthesis and its component is coded by CRD1 gene[397]. Knockout mutant of CRD1 led to retarded growth, defects in chloroplast development and repression of genes coding for LHCI and reduced photosynthetic activity, indiciating damage to PSII reaction centres[397]. CHLH catalyses the insertion of Mg^{2+} ion into the protoporphyrin IX and is probably a positive regulator of ABA signalling in seed development, seedling growth and stomatal opening[398]. Chlorophyll synthesis was also found inhibited in wheat, cucumber and rice under low temperature stress[399–401]. However, the rice tolerant variety Japonica showed greater accumulation of chlorophyll than sensitive Indica rice variety plants[400]. Therefore, chlorophyll accumulation has been suggested to be associated with increased plants cold tolerance ability[21]. The mechanism through which chlorophyll accumulation may increase cold tolerance is not yet known, however, downregulation of genes involved in chlorophyll biosynthesis may possibly increase basil plants susceptibility to chilling conditions.

Other biological processes including Sulfuric amino acid biosynthesis and response to nematode were annotated to genes such as Cobalamin-independent synthase family protein(ATMS1) and Pyridoxal phosphate (PLP)-dependent transferases superfamily protein (MTO1) involved in biosynthesis of methionine amino acid and facilitator superfamily protein, dicarboxyate transporter (DIT1) and multi drug associated protein

4 (ABCC4). However, role of those genes in relation chilling temperatures are not yet known.

The downregulation of genes involved in preventing accumulation of ROS and protection of photosystem from oxidative stress would be disadvantageous to LR:FR light pre-treated plants under chilling stress. However, several other biological processes cell redox process, cellular homeostasis and oxidation-reduction have been found enriched in response to LR:FR light where genes such as glutaredoxin-C1, monothiol glutaredoxin -S2, peroxiredoxin-2F and cytochrome P450 90C1 with roles in scavenging of ROS were among permanently upregulated genes. Hence, plant related with LR:FR light may be disadvantaged by temporary loss of oxidoreductase, glycerophosphophosphate-3-dehydrogenase and lipocalin genes but at the same time are also advantaged by permanent upregulation of genes such as lutaredoxin-C1, monothiol glutaredoxin -S2, peroxiredoxin-2F and cytochrome P450 90C1. Thus, it may be possible that permanent upregulation of genes are able to provide the protection that was reduced by temporary downregulation of genes annotated to photosynthesis light harvesting process.

6.2.7 Biological processes enriched among genes that showed upregulation in response to LR:FR light at ZT6 and ZT10 but HR:FR light shows lower gene expression than basal level at ZT10.

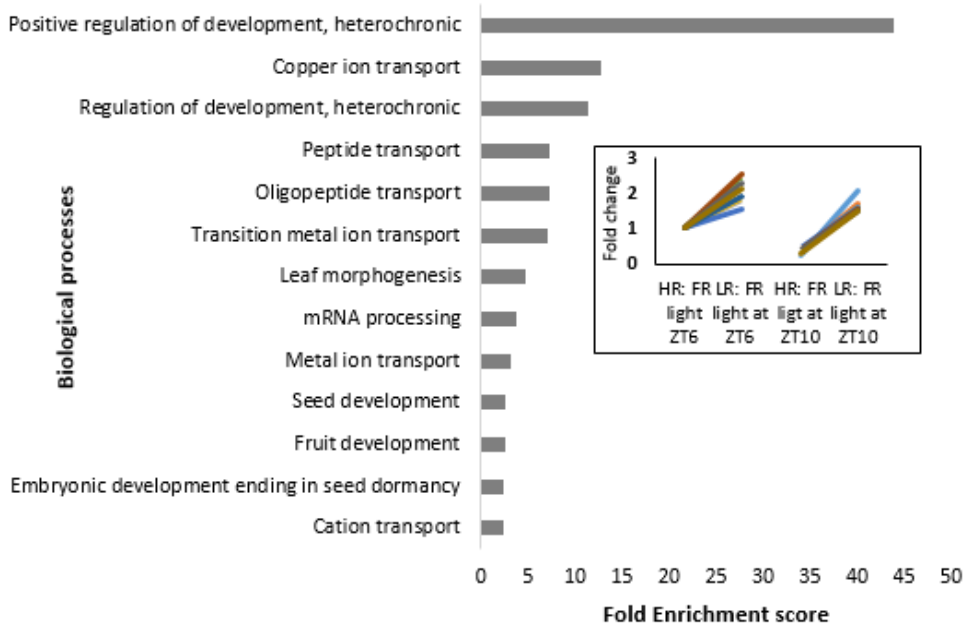


Figure 6.7 Shows significantly enriched biological processes (p<0.05 based on Fisher's Exact p value by EASE) among genes that showed downregulation in response to HR:FR light at ZT10 but were permanently upregulated by LR:FR light treatment. The icon of the gene expression pattern was added to show the behaviour of the genes being discussed.

13 different biological process were found enriched among genes that showed downregulation to HR:FR light at ZT10 but were upregulated by LR:FR light at ZT6 and ZT10. The biological processes include positive regulation of development, copper ion transport, regulation of development, peptide transport, oligopeptide transport, transition metal ion transport, leaf morphogenesis, mRNA processing, metal ion transport, seed development, fruit development, embryonic ending in seed and cation transport (Figure 6.7) (For details of genes annotated to each enriched biological

processes, see appendix 6.11). Peptide transport and oligopeptide transport were co-annotated to genes such as oligopeptide transporter 7, probable nicotinamide transporter YS16 and oligopeptide transporter 6. Among these gene oligopeptide transporter 7 and oligopeptide transporter 6 function to transport peptides across the cellular membrane in an energy dependent manner where as probable metal-icotanamide transport YS16 may be involved in transportation of nicotnamide-chelated metals. Peptide transport is one of the main ways through which cells take up nitrogen from its surrounding environment for metabolism of amino acids and growth [402]. Once the peptides are internalised they are hydrolysed by peptidases and used as a source of amino acids, nitrogen and carbon [403].

According to Koh *et al.*[403] peptide transport in higher plants has received little information compared to prokaryotes and animals and their enrichment role in chilling tolerance is not yet known. However, peptide transport across the membrane has been suggested to be a more efficient distribution of nitrogen than the transport of individual amino acids[403]. Hence, upregulation of these peptide transporters by LR:FR light treatment could possibly support plants growth during chilling stress when photosynthesis and growth is normally reduced in chilling sensitive plants [87].

6.2.8 Biological processes enriched among genes that showed upregulation in response to LR:FR light at ZT10 alone.

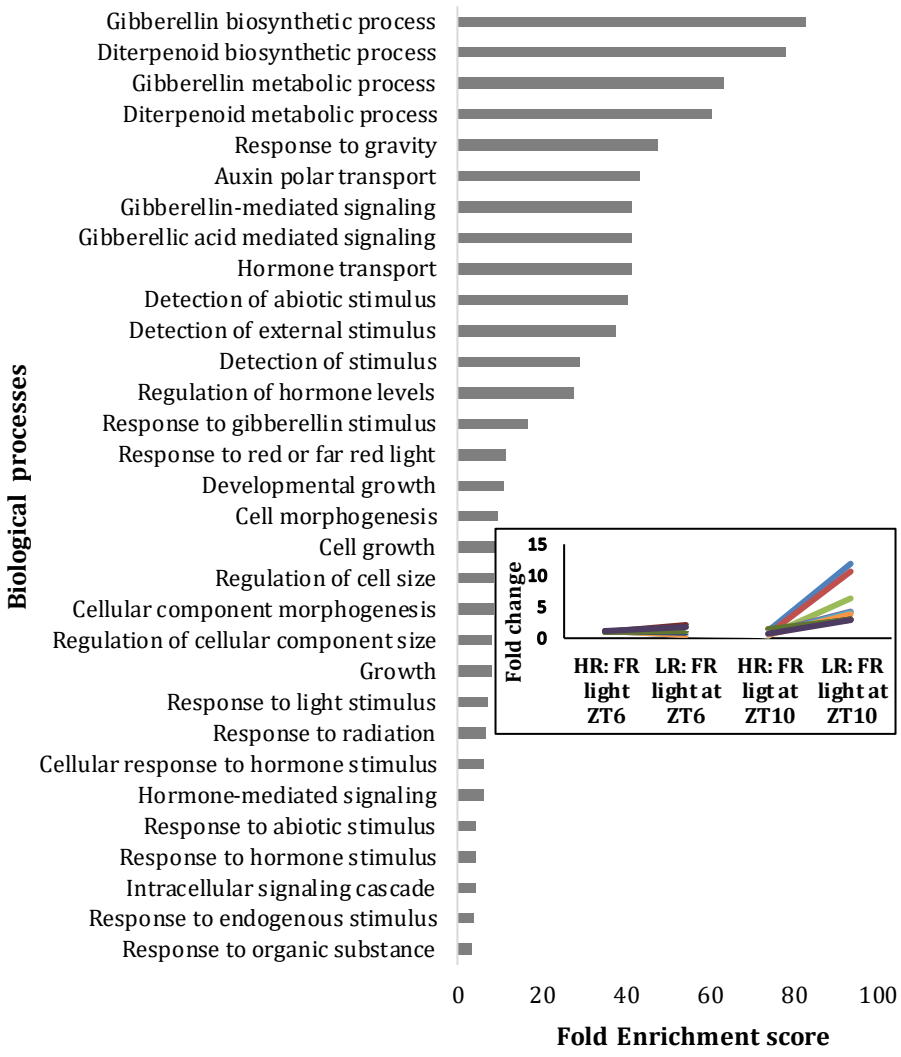


Figure 6.8 Significantly enriched biological processes (p<0.05 based on Fisher's Exact p value by EASE) among upregulated genes showing 1.5 fold or higher expression in response to LR:FR light at ZT10. The icon of the pattern is show to display the behaviour of the genes being discussed.

31 different biological processes were found enriched among genes that showed upregulation to LR:FR light at ZT10 only and were unresponsive to LR:FR light at ZT6 and HR:FR light at ZT10 (Figure 6.8) (details of genes annotated to each biological processes can be found in appendix 6.12). Among these 31 different biological processes, gibberellin biosynthetic process, gibberellin metabolic process, diterpenoid

biosynthetic process, diterpenoid metabolic process, giberelln mediated signalling, gibberellic acid mediated signalling, detection of abiotic stimulus and response to abiotic stimulus, response to radiation, response to light stimulus, intracellular signalling cascade, response to red or far red light, response to hormone stimulus, response to organic substance and response to endogenous stimulus were found co-annotated to genes gibberellin 20 oxidase 1, gibberellin oxidase 2, transcription factor PIF3 and phytochromes A[404]. *Achard et al.*[404] showed that the CBF activation results in upregulation of genes coding for gibberellin 2 oxidase genes. Giberellin 2 oxidase causes a decrease in active gibberellin and increase in the levels of DELLA proteins to inhibit growth. This inhibition in the growth of the plant mediated by CBF genes have been shown to cause increased tolerance of the plant to freezing temperatures in *A. thaliana*. Added FR light from LR:FR light causes increased phyA signalling that from the study by Wang *et al.*[261] has been suggested to play a role in positive regulation of cold tolerance in plants. Hence, a delayed upregulation of genes such as gibberellin 2 oxidase and phyA may possibly increase LR:FR light treated basil plants ability to adapt to chilling temperature.

6.3 Analysis of differences in gene expression following 24 h of chilling stress in plants grown with or without LR:FR light pre-treatments

The basil pre-treated with or without LR:FR light and subsequently stored under chilling temperature and continuous dark conditions for 24 h were RNA sequenced (Figure 6.9). The sequenced transcriptomes were analysed to investigate the molecular changes in chilled basil pre-treated with Vs without LR:FR light (Figure 6.9). 3405 genes were differentially expressed, among which 924 genes (27%) were upregulated by 1.5 folds or higher and 2481 genes were (73%) were downregulated by 0.6 folds or less, representing chilling temperatures to have had a greater suppressive effect on basil's transcriptome.

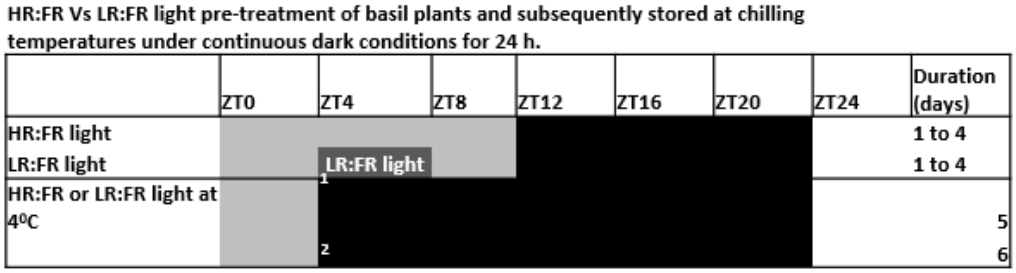


Figure 6.9 Shows pre-treatment of basil with HR:FR or LR:FR light at 12 h photoperiod for four days. The LR:FR light was given between time points ZT4 – ZT8 for four days. On the fifth day basil plants were transferred at ZT4 (1) from their light treatments to either chilling or ambient temperature under continuous dark conditions for 24 h. The basil samples were harvested on the sixth day at ZT4 (2) and were immediately frozen in liquid nitrogen for subsequently storage at -80^{0}C for molecular experiments or fresh material was used for physiological experiments.

6.3.1 Enrichment of biological processes among genes that showed higher expression following 24 h of chilling stress in plants grown with versus without LR:FR light pre-treatment.

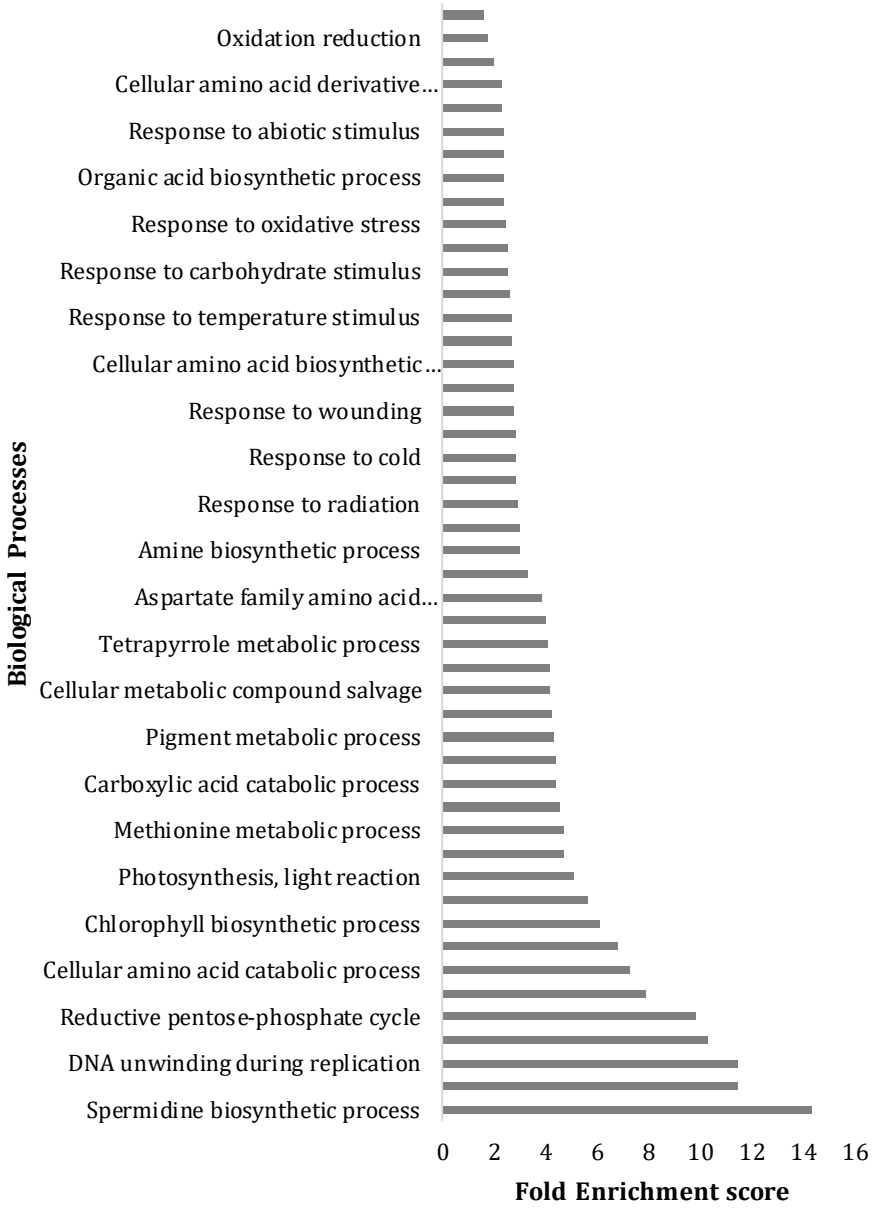


Figure 6.10 Enriched biological processes (p<0.05 based on Fisher's Exact p value by EASE) among genes showing 1.5 fold or higher expression following 24 h of chilling stress in plants grown with versus without LR:FR light pre-treatment.

195 different biological processes were found enriched among genes that showed 1.5 fold or higher expression following 24 h of chilling stress in plants grown with or

without LR:FR light pre-treatment, (Figure 6.10) (P value <=0.05 and enrichment score >1.5, for details of genes annotated to each biological process, see appendix 6. 13). Among these biological processes, the enriched biological processes discussed in this present study are, spermidine biosynthetic process, reductive pentose-phosphate cycle, cellular amino acid catabolic process, chlorophyll biosynthetic process, photosynthesis light reactions, carboxylic acid catabolic cellular process, response to cold, response to oxidative stress, response to abiotic stimulus, response to metal ion and oxidation-reduction.

The most enriched biological process spermidine biosynthetic process was annotated by genes including S-adensoylmethionine carboxylase, arginine decarboxylase 1 and arginine decarboxylase 2 are all involved in the biosynthesis of polyamines[405,406]. Polyamines such as spermine and spermidine are polycations and interact with negative charged molecules such as DNA, RNA and proteins[407]. Mutants of *E.coli* strains in polyamines showed greater susceptibility to damage by oxidative stress and are, therefore, suggested to be involved in protection of the cells from oxidative damage caused by hydrogen peroxide[408,409]. Due to the nature of polyamine molecules and their close association with the DNA during condensation, particularly by spermine, polyamines are suggested to be the primary protectors of DNA from damage by oxidative stress[410,411]. Spermine is considered a more potent polyamine than spermidine as spermine has been found a more active scavenger of ROS[412,413]. Polyamines also been found to induce conformational changes in DNA molecule to stabilise and protect it from oxidative damage under stress conditions[414,415]. Upregulation of genes (S-adensoylmethionine carboxylase, arginine carboxylase 1 and arginine carboxylase) annotated to the most enriched biological process (spermidine biosynthetic process) by pre-treatment of LR:FR light indicates the importance of polyamines roles in plants response to chilling stress**.** The role of polyamines in

protection of cells from oxidative stress also indicates the importance of ROS accumulation and their effects on plant cells injury under chilling stress. Hence, a possibly induced synthesis of genes involved in spermidine biosynthesis shows that basil's transcriptome was inducing mechanisms to adapt to chilling stress.

Genes involved in chlorophyll biosynthesis (glutamyl t-RNA reductase, CRD1 and CHLH) or photosynthesis, light reactions (LHCA2, LHCA3, LHCB and LIL3.2) were downregulated temporarily by LR:FR light before chilling treatment, however, the same genes were found upregulated after chilling treatment. LIL3.2 and LHCA3 play a role in photoprotection and cause accumulation of carotenoids [396,416] while others can cause increased chlorophyll synthesis and may play a role in increasing basil's ability to tolerate chilling temperature.

The response to cold biological process included genes such as catalase 2, catalase 3, HSP70, WRKY transcription factor 33, MAPK3, uncharacterised calcium binding protein, arginine decarboxylase. The response to cold along with reductive pentose-phosphate cycle included products of genes involved in the Calvin cycle such as glyceraldehyde-3-phosphate dehydrogenase a, glyeraldehyde-3-phosphate dehydrogenase b, phosphoribulokinase, ribulose bisphosphate carboxylase, and phosphoglucomutase. The genes annotated to cold coded for proteins with a wide range of properties including antioxidative, chaperone, be able to initiate signalling cascades and induce expression of downstream target genes. The properties of the products of genes annotated to response to cold are suitable for providing protection against damage by chilling temperature stress. Catalase 2, catalase 3 are antioxidative enzymes while HSP70, WRKY transcription factor 33, MAPK3, an uncharacterised calcium binding protein and arginine decarboxylase are transcripts whose expression is known to be induced during environmental stress including cold stress [277,417–419].

ROS in their increased content during chilling stress can also function as second messengers where they can cause activation of calcium channels and trigger signalling cascades including MAPK signalling[420]. The activation of MAPK3, in turn, can cause activation of WRKY 33 transcription factor. The MAPK3 signalling cascade and WRKY 33 transcription factor can both induce transcription of stress responsive genes that results in increased plants tolerance towards particular environmental stress or multiple environmental stress [421]. MAPK3 and WRKY 33 transcription factor were expressed at higher levels in the basil pre-treated with LR:FR light prior to 24 h of chilling treatment. Thus, it may be possible that their increased transcription may in part play a role in basil's increased tolerance towards chilling conditions.

Genes such as glyceraldehyde-3-phosphate dehydrogenase A, glyceraldehyde-3-phosphate dehydrogenase B and phosphoribulokinase drive the process of photosynthesis dark reactions. The fact that they are expressed at higher levels in LR:FR pre-treated plants may indicate that LR:FR pre-treatment may prevent such extreme downregulation of photosynthetic genes in the subsequent cold treatment. Glyceraldehyde-3-phosphate dehydrogenase also plays roles in suppressing the production of hydrogen peroxide and ROS-mediated cell death[422] so its high expression level may also be part of an improved cold tolerance.

As previously mentioned, chilling temperature causes increased production of ROS and can act as second messengers but in large quantities can become toxic to the cell. ROS levels are also increased in large quantities in plants under metal ion stress [380]. Hence, it is logical that a large proportion of the differentially expressed genes annotated to the enriched biological processes response to metal ions were genes with antioxidative properties. They include glycerladehyde-3-phosphate dehydrogenase A and glyceraldehyde -3-phosphate dehydrogenase B, catalase 1, catalase 3, a probable phospholipid hydroperoxide glutathione peroxidase 6, thioredoxin H-type 5 and

isoflavone reductase homolog P3. Other genes in the same category include heat shock protein 70 kDa protein 1, heat shock protein 70 kDa protein 2, heat shock protein 70 kDa protein 3, and dynamin-related protein 1E. Similarly, many ROS-related genes are found among differentially expressed genes annotated to the enriched biological process categories, response to oxidative stress, as well as oxidation-reduction. These include catalase 1, catalase 2, catalase 3, arginine decarboxylase 1, arginine decarboxylase 2, peroxidase, superoxide dismutase, thioredoxin H-type 5, glutathione peroxidase 6, glutamyl-tRNA reductase 1, glutamyl t-RNA reductase 2, peroxidase 47, nitrate reductase , nitrate reductase 2, PGR5-like protein A and PGR5- like protein 1B. Their increased expression in basil pre-irradiated with LR:FR light might suggest that those plants will consequently possess greater protection against cellular injury from oxidative stress associated with chilling than plants not pre-treated with LR:FR light. Again, the differential expression of these genes may be related to the enhanced chilling tolerance in LR:FR pre-treated plants.

6.3.2 Enrichment of biological processes among genes that showed lower expression following 24 h of chilling stress in plants grown with versus without LR:FR light pre-treatment.

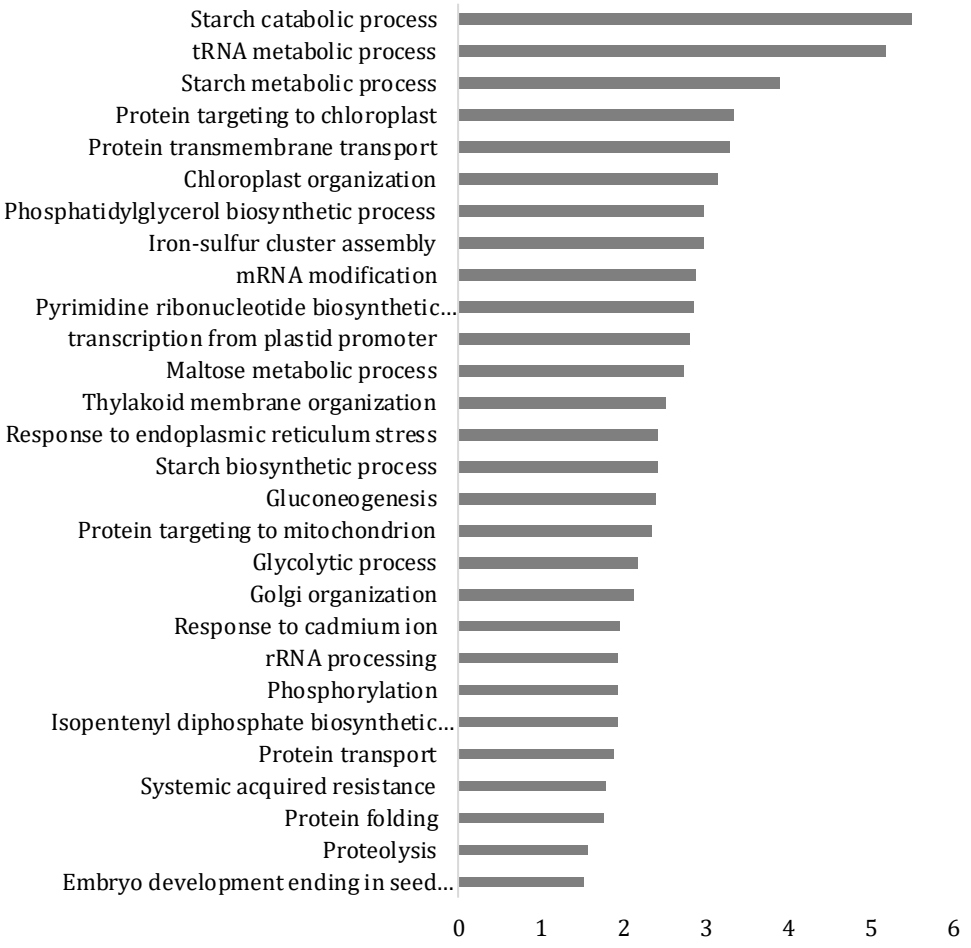


Figure 6.11 Significantly enriched biological processes (p<0.05 based on Fisher's Exact p value by EASE) among genes showing 1.5 fold or higher expression following 24h of chilling stress in plants grown with versus without LR:FR light pre-treatment.

111 different biological processes were found significantly enriched among genes that showed low expression in basil plants post chilling stress but pre-treated with versus without LR:FR light. Among these 111 genes, the biological processes discussed here include starch catabolic process, starch metabolic process, protein targeting to chloroplast, protein transmembrane transport, chloroplast organization, phosphatidylglycerol biosynthetic process, iron-sulphur cluster assembly, starch

biosynthetic process and proteolysis. (Figure 6.11) (Genes annotated to each biological processes can be found in appendix 6.14).

Starch metabolism appears to have downregulated in chilled basil pre-treated with LR:FR light. A decline in the starch metabolism has also been observed in *Lolium temulentum* when under chilling temperatures. The biological process starch catabolism or starch degradation were annotated to the genes including a-glucan water dikinase (SEX1), a-amylase like 3 (AMY3), like SEX4 (LSF1), and plastidic glucose translocator (pGlcT) or DNAse I-like superfamily protein, starch branching enzyme 2.1 (SBE2.1) and SBE2.2 genes, respectively. Catabolism of starch leads to accumulation of carbohydrates such as glucose and fructose and is associated with increased cellular tolerance towards low temperatures[285]. Hence, increased expression of genes associated to starch catabolism such as α-glucan water dikinase (SEX1), α-amylase like 3 (AMY3), like SEX4 (LSF1), and plastidic glucose translocator (pGlcT) could have contributed to plants cold tolerance[285,423–425] and their downregulation is therefore likely to be disadvantageous to basil plant cells under chilling stress. On the other hand starch synthesis would cause storage of glucose into polymers of amylose or amylopectin in the chloroplast[426] and thereby reduce glucose availability in the cytosol. Thus, genes downregulation of genes associated with starch synthesis may be beneficial for basil's cell's survival and protection against damage by cold.

Chloroplast is involved in lipids, amino acid, nitrogen, sulphur metabolism and photosynthesis and is therefore an important organelle. Hence, *A. thaliana* consist of mechanisms that maintain chloroplast homeostasis and includes importing of the nuclear encoded chloroplast proteins. Chloroplast destined protein carry a distinguished N terminal transit peptide (TP) and is recognised by members of two family receptors known as Toc GTPase receptors (TOC132 and TOC159)[427]. They are primarily responsible for importing photosynthetic pre-proteins but none of the single

mutants in *A. thaliana* showed any strong change in visible phenotypes[428]. Hence, the impact of TOC159 and TOC132 downregulation in basil plant cells will probably not be detrimental on their development of chilling tolerance. Outer membrane OMP85 family protein (TOC75) is expressed throughout seed development, involved in assembly and insertion of beta-barrel proteins into the outer membrane. OMP85 knock out mutants were defective in etioplast and were less efficient in de-etiolation than wild type plants[429].

The transmembrane target proteins was annotated with genes located on the membrane and involved in transportation of molecules across the membrane. These genes include ABC ATP binding casette (ABCB1), auxin efflux carrier family protein, major facilitator superfamily with SPX and yellow stripe like 1 (YSL1) and were involved in either transportation of auxin, efflux of auxin from the cell or transportation of Pi into the vacuole, respectively[430–432]. Mutants of either ABCB1 or SPX resulted in either dwarfism or lower Pi storage than wild type, respectively[430,432]. The mutants of these genes show that the downregulation of transporters were associated with transportation of molecules, compounds and metals whose role is probably not essential for plants development to cold tolerance. In fact, accumulation of auxin or sequestration of Pi may possibly reduce a plants ability to tolerate chilling conditions. A build up of auxin may promote elongation and cause the nutrients to be utilised in unnecessary growth that could instead be used to provide protection to the cells against damage by chilling temperature. Chilling temperature can cause metabolism to slow down and may thereby reduce the amount of Pi in cell, therefore, sequesteration of Pi into the vacuole under such conditions may lead to production of ROS and cause oxidative stress. Thus, downregulation of ABCB1 and SPX may support basil cells acclimation to chilling conditions.

Genes annotated to chloroplast organisation and iron sulphur assembly include alternative oxidase family protein (IM), CLPP6, EGY1 and fructokinase-like 2 A (FLN2) where the proteins coded by the genes were found important for chloroplast biogenesis, development and integrity[433–436]. Mutants of these genes showed defects in chloroplast development and change in phenotype of plant from those of wild type. For instance, loss of ClpP6 caused reduced growth and impaired chloroplast development[434]. The egy1-1 mutant showed reduced chlorophyll accumulation, reduced granal thylakoids and poorly developed lamellae[435]. *A. thaliana* lacking IM showed an increased rates of photosynthesis, increased activities of Rubisco and accumulation of sucrose[437] while *fln2* genes resulted in reduced growth and development of *A. thaliana*[436].

Genes associated with chloroplast organisation appear important for development of chloroplast and growth of plants. However, basil plants stored under cold conditions for 24 h were fully grown and mature, therefore, downregulation of genes described above may not have a detrimental effect on basil's survival under chilling temperatures.

Downregulated genes involved in the degradation of proteins and associated with the biological process proteolysis include CLPR1, CLPR6, DEG2 and AAA ATPase where all the genes are considered essential for chloroplast development and function in *A. thaliana*[434,438–440]. CLPR and CLP6 are ATP dependent protease and involved in degradation of stromal enzymes including Rubisco and fructose bisphosphate aldolase enzymes[434,438]. Loss of CLPR1 and antisense line of CLPP6 and also showed a slow growth with chlorotic leaves in *A. thaliana* but recovered later upon maturation[434,438]. DEG2 carries out degradation of LHCB6 in response to short duration stress[439] while FtsHi4 contributes to the degradation of LHCB3[441].

Downregulation of genes included in the proteolysis biological process may support survival of basil plants under chilling conditions. It would prevent degradation of components essential for carbon fixation ad thereby promote cryoprotection of cell against damage by cold.

From the analysis of enriched biological process among downregulated genes of basil pre-treated with LR:FR light and followed by storage at chilling temperature, it appears that the genes were involved in inhibiting storage of soluble sugars, inhibiting proteolysis, growth and development of the plant and chloroplast. Under normal physiological conditions, downregulation of such genes is likely to be disadvantageous for the plants, however, under stress conditions, downregulation of such processes may be necessary to promote survival of the plant. In addition, when basil was stored under chilling conditions it was mature and ready for transportation to the supermarkets, therefore, downregulation of genes involved in chloroplast synthesis, growth and development of plants may not be detrimental for its survival under chilling conditions for 24 h. Thus, basil plants with reprogrammed transcriptome after having been treated with LR:FR light and subsequently cold conditions seems to have induced changes that would allow it to show better tolerance against damage by cold than plants not pre-treated with LR:FR light.

6.4 The visualisation of genes that showed higher and lower expression that are involved in the metabolic pathways of the primary metabolism following 24 h of chilling stress in plants grown with versus without LR: FR light pre-treatment.

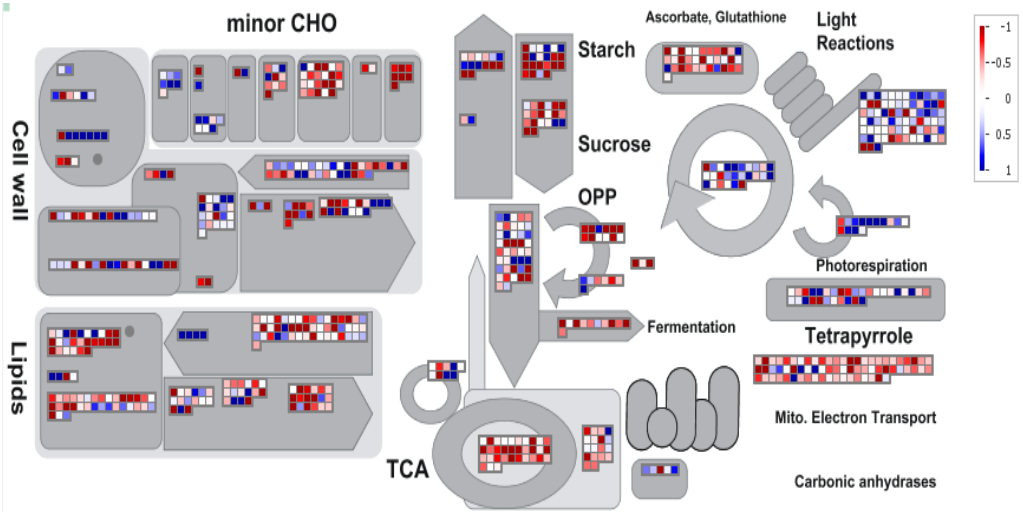


Figure 6.12 Image obtained from Mapman exhibiting an overview of the gene expression changes occurring in the pathways of the primary metabolism where the genes showed either higher or lower expression following 24h of chilling stress in plants grown with versus without LR:FR light pre-treatment. The gene expression change shown in the image is scaled from -1(red) to +1(blue) where -1 represents low gene expression by 0.5 or less and +1 represents high expression by 2 fold or higher.

From the appearance of the metabolism overview it appears that there was greater downregulation of genes rather than upregulation in metabolic pathways of primary metabolism such as lipid metabolism, carbohydrate metabolism that includes carbohydrate catabolic process such as starch and sucrose degradation while those involved in the carbohydrate biosynthetic process were upregulated including light reactions, Calvin cycle, starch and sucrose synthesis (Figure 6.12). Other important

metabolic pathways also important for cellular function including tetrapyrrole and ascorbate and glutathione antioxidant defense system were observed to be downregulated in response to LR:FR light irradiation under chilling conditions.

In lipid metabolism, lipid synthesis was mostly downregulated along with lipid modification and degradation. However, enzymes involved in the desaturation of fatty acids such as FAD2, FAD3, FAD5 and FAD8 that play a role in delaying the transition of the membrane from liquid phase to solid[442] were upregulated in basil plants response to LR:FR light irradiation before being exposed to cold. The downregulated genes coding for enzymes in lipid degradation include putative lipase, lipase class 3 family protein and hydrolase whose upregulation is incompatible with cold acclimation. The lipases function to digest the phospholipids and as the membrane integrity is usually compromised upon chilling stress, increased degradation of the phospholipids is incompatible with cold acclimation of plants[174]. Therefore downregulation of genes involved in lipid metabolism is probably important for resistance of membrane from damage induced by chilling stress[174].

Photosynthetic inhibition at times of chilling stress mainly occurs due to thylakoid lipid peroxidation occurring in response to increased oxidative stress caused by chilling conditions, due to reduced transportation of sugars from leaves causing photosynthesis to be inhibited via negative feedback regulation, inactivation of the Rubisco enzymes or due to inhibition of starch and sucrose synthesis that leads to Pi limitation needed by calvin cycle[152]. From the metabolic pathway analysis of light reactions and Calvin cycle, it appears that plants treated with LR:FR light is likely to have increased resistance of photosynthesis against suppression from chilling temperatures. This is likely to have occurred because genes coding for enzymes involved in light reactions and Calvin cycle driving the process of photosynthesis were found upregulated[327,443]. Key genes upregulated among the light reactions include

chlorophyll a-b binding family protein, light harvesting complex chlorophyll b binding, light harvesting complex of photosystem II 5, photosystem II subunit X, PSBY, serine threonine protein kinase 7 and proton regulation gradient 5 while those upregulated in calvin cycle pathway includes putative phosphoglycerate kinase, putative phophoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase, sedoheptulose-bisphosphatase, phosphoribulokinase, Rubisco activase and phosphoglycerate kinase. At times of chilling stress plants are likely to imbalance between the amount of light energy absorbed and the amount used by the plant metabolism[106]. Thus, an increase in the pathway of calvin cycle in plants can increase the electron consumption capacity and thereby reduce the imbalance between the light energy absorbed and light energy converted into chemical energy[326]. This one of the mechanism through which plant adapts to the environment and prevent suppression of photosynthesis during chilling or light stress. Hence, an upregulation of genes involved in the calvin cycle observed in this present study in response to LR:FR light pre-treatment may also play a role and prevent downregulation of photosynthesis under chilling stress. According to He *et al.*[137] suppression of photosynthesis in spinach plant during chilling stress was prevented by accumulation of polyamines in the cell that limits adverse effects of ROS on cell and cellular components. Arginine decarboxylase 1 and arginine decarboxylase 2 that are involved in the synthesis of polyamines were upregulated in basil plants pre-treated with LR:FR light. Hence, it may be that the possibly increased polyamine content of the cell potentially by arginine decarboxylase 1 and arginine decarboxylase 2 could in part have prevented photosynthetic inhibition from chilling stress. Photosynthesis may also have been prevented in its suppression by chilling stress due to upregulation of key genes involved in starch and sucrose synthesis such as starch synthase, starch synthase 2 and fructose-2, 6-bisphosphatase. Upregulation in starch and sucrose synthesis can prevent Pi depletion.

Factors involved in conferring cold acclimation apart from increased expression of COR and stress related gene products is accumulation of sugars, particularly sucrose. In spinach and also in sweet cherry the enzymes such as sucrose phosphate synthase involved in sucrose synthesis were found upregulated while those involved in sucrose degradation such as invertase and sucrose synthase were found downregulated during low temperature stress[444,445]. Sucrose was the most abundant sugar compared to fructose and glucose accumulation in spinach during cold acclimation. Similar results were found in the transcriptomic profile of basil pre-treated with LR:FR light that after exposure to chilling conditions showed increased expression of genes involved in sucrose synthesis such as fructose-6-bisphosphatase and shows downregulation of invertase. The sucrose metabolism reprogramming in response to LR:FR light treatment suggests for sucrose content in the cell to have possibly accumulated and thereby provide increased resistance to cold temperatures.

Antioxidant defense system analysed from the mapman pathway analysis tool showed for ascorbate and glutathione cycle to be mostly downregulated. Ascorbate is an important antioxidant molecule that requires monodehydroascorbate reductase and dehydroascorbate reductase for its regeneration and their upregulation confer cold tolerance in plants[446]. However, genes coding for proteins involved in ascorbate and glutathione cycle in response to LR:FR light appeared to be mostly downregulated that includes genes such as monodehydroascorbate reductase, putative, L ascorbate oxidase, putative, L-ascorbate oxidase, monodehydroascorbate reductase and L-galactose dehydrogenase. An increased activation of ascorbate and glutathione cycle was found in the leaves of cucumber upon its exposure to chilling stress and thereby resulted in its increased tolerance to chilling temperatures[447]. Hence, downregulation of ascorbate and glutathione cycle antioxidative system is likely not be beneficial for basil's survival under chilling stress. It is however important to note that plants in

response to oxidative stress consist of enzymatic and non-enzymatic antioxidant system that are exclusive of ascorbate and glutathione cycle. Those antioxidants include superoxide dismutase, catalase, peroxidase and glutathione reductase[105] that were found upregulated in basil pre-treated with LR:FR light before their exposure towards chilling stress. There are also other enzymes that may directly or indirectly play their role in scavenging of ROS such as glyceraldehyde-3-phosphate dehydrogenase A and glyceraldehyde-3-phosphate dehydrogenase B, arginine decarboxylase 1, arginine decarboxylase 2 and HSP's that limit the adverse effects of ROS on the cell[277,279,422]. Hence, although ascorbate and glutathione cycle are downregulated in plants pre-treated with LR:FR light and is probably not favourable for basil plants cold acclimation process, there are however other antioxidative mechanisms that are upregulated and may facilitate the alleviation of oxidative stress in basil cells under chilling stress.

The tetrapyrole metabolic pathway revealed for basil treated with LR:FR light to show downregulation of genes involved in the cytochrome electron transport chain while alternative oxidase was upregulated. These results are similar to the findings of chilling sensitive maize upon exposure to chilling temperature (5 days at 5 °C) to cause dramatic decline in cyt pathway and an increase in alternative oxidase activity that 60% of the respiration was occurring through alternative oxidase [158]. Alternative oxidase plays a role in providing tolerance to the plants in response to various environmental stresses such as low temperature, drought and nutrient deficiency by causing direct reduction of ubiquinone to water and resulting in suppression the production of ROS54. In fact, alternative oxidase increased transcript expression is considered as a general marker of mitochondrial dysfunction and mitochondrial oxidative stress as alternative oxidase is also induced by increased ROS expression [158]. Hence, increased expression of alternative oxidase in basil treated with LR:FR suggests that the

mitochondrial function suggests that mitochondrion under chilling stress was likely to have experienced oxidative stress and possibly affected by chilling temperature. However, the increased expression of alternative oxidase represents acclimation response of the plant towards chilling induced mitochondrial dysfunction when pre-treated with LR:FR light.

6.5 Conclusion

This chapter was an attempt to pre-screen the changes occurring in basil at gene expression level when pre-treated with LR:FR light compared to plants treated with HR:FR light. Possible suggestions have been proposed in this chapter that may explain basil plants differential response towards chilling temperatures. The upregulation of genes including KIN11, acyl co a oxidase 1 - 5 and transcription factor phyA in LR:FR light treated basil plants at ambient temperatures, indicate a possible activation of phyA, increased ABA and JA signalling and their potential involvement in basil's increased tolerance to chilling temperatures. It was also interesting to note that many of the enriched biological processes among upregulated genes by LR:FR light were involved in detoxification of ROS (catalase 2, catalase 3, arginine decarboxylase 1, arginine decarboxylase and peroxidases). Role of ROS in chilling injury was also indicated in the first RNA sequencing experiment, when many of the genes found upregulated were involved in suppression of ROS formation. Thus, it seems that the accumulation of ROS during chilling stress play a major role in inducing chilling injury in plants. Therefore, it is possible that basil pre-treated with LR:FR light containing increased expression of antioxidants before being exposed to cold may have shown greater tolerance, as it was better equipped with defense mechanism against chilling induced oxidative stress than plants pre-maintained in HR:FR light (control). Basil pre-treated with LR:FR and subsequently stored at chilling temperatures showed reversal in expression of genes involved in chlorophyll synthesis, stabilisation of PSII under

stress conditions and calvin cycle (LIL3.2, LHCA3, glyceraldehyde-3-phosphate dehydrogenase a and glyeraldehyde-3-phosphate dehydrogenase b). Genes involved in chlorophyll synthesis, stabilisation of PSII and Calvin cycle were upregulated in basil after storage at cold temperatures but pre-treated with LR: FR light, however, the same genes were downregulated at ambient conditions. Genes involved in chlorophyll synthesis and Calvin cycle can increase a plants photosynthetic capacity and also protect the photosynthetic apparatus from oxidative damage. Thus, their reverse expression after cold treatment in LR: FR light pre-treated basil makes them genes of interest for further investigation. By showing a change in their expression, the genes involved in chlorophyll biosynthesis and calvin cycle show that they are responsive to cold and their reversed expression indicates their role to be possibly involved in cold tolerance. Thus, investigation in the roles of these genes in basil response to cold temperatures can be investigated as part of future work. Transgenic mutants carrying gain-of-function mutation of either LIL3.2, LHCA3, glyceraldehyde-3-phoshate dehydrogenase a or glyceraldehyde-3-phoshate dehydrogenase b, can be created and their impact on basil's ability to tolerate chilling temperatures investigated.

In this present study, basil plants were harvested at ZT6 and ZT10 on the fourth day of periodic LR:FR light treatment and then on the sixth day after their storage at chilling temperatures. It would therefore be of interest to follow other time points and particularly those in between of ZT10 fourth day of light treatment and the sixth day of harvest, after basil's storage chilling temperature. This would identify whether the basil's transcriptomic changes observed at time points ZT10 were in fact "permanent" or not. CBF genes peak in their expression at ZT8, therefore, basil material can be harvested at time points ZT4, ZT8, ZT12 on the fourth day of LR:FR light treatment and again at ZT4 on the fifth day just before transfer of basil to chilling or ambient temperatures. This experiment can also identify whether the genes found upregulated

after storage at cold temperatures were already induced as part of basil's preparation to chilling stress or a consequence of chilling stress.

7 GENEREAL DISCUSSION AND CONCLUSION

The overall aim of the project was to establish ways of modifying current growing conditions of basil to enhance chilling tolerance in chilling sensitive basil. This was approached by addressing four main areas that included establishing the damage experienced by the plant in response to chilling conditions at the physiological and molecular level to identify cellular components, biological processes and metabolic pathways that are most affected by chilling temperatures. These changes helped to identify areas that could offer ways to improve plant tolerance towards chilling conditions. The gene expression changes associated both with chilling damage and those associated with plants treated to enhance chilling tolerance are compared and these changes used to try and elucidate the possible mechanisms employed to bring about the cold acclimation effect on chilling sensitive basil plants.

It is clear from the molecular and physiological experiments that basil is sensitive to chilling conditions as, in response to those chilling conditions, damage to the cellular components were seen that were typical of a chilling sensitive plant. These included reduced fresh weight of the leaves, and damage to cell membranes as manifest by increased lipid peroxidation and electrolyte leakage, possibly brought about by increased levels of ROS and reduced levels of antioxidants. Damage to the photosynthetic apparatus was also detected by a reduction in the delayed chlorophyll fluorescence yield. Analysis of RNAseq data revealed that downregulation of genes predominated over upregulation, suggesting that metabolic processes were, in general, reduced as a result of chilling temperatures. These data have led to the identification of various biological processes and metabolic pathways as being involved in the tolerance or susceptibility of basil to chilling temperatures. The gene ontology enrichment analysis on cellular component category confirmed that the plasma membrane and chloroplast were the main sites most affected by chilling temperatures, thus confirming what was suggested by physiological experimental

results. Indeed, most differentially (up or down) regulated genes, in response to chilling conditions, were annotated to either the plasma membrane or chloroplast. For differentially regulated genes, these components were among the top cellular component terms showing enrichment with respect to the genome as a whole, showing that they play important roles in the plant's response to chilling temperatures.

The biological processes associated with upregulated genes in basil treated with chilling temperatures were found to be similar to those previously reported to be associated with other environmental stresses, for example abiotic stimuli, response to metal ion stress, salt stress and osmotic stress. These biological processes share common effects that include dehydration and oxidative damage that may be detrimental for the plant's development. The genes that were strongly upregulated within these enriched biological processes included arginine decarboxylase 1, arginine decarboxylase 2, aconitate hydratase 1, aconitate hydratase 2, fructose-2, 6-bisphosphatase and peroxiredoxin-2 BAS1, which are associated with antioxidative roles. Upregulation of genes with roles in quenching of ROS further adds to the suggestion that basil plants exposed to chilling stress are likely to have suffered from oxidative stress. Moreover, the induction of these genes could possibly have occurred, in part, for adaptation to oxidative damage induced by chilling stress. Among the downregulated genes in basil in response to cold, one of the enriched biological processes was oxidation reduction that was annotated with 89 genes with antioxidative roles. These included ferredoxin, thioredoxin, nitrate reductase and peroxidase. The downregulated genes also included those of the ascorbate and glutathione cycles, where some of the downregulated genes included monodehydroascorbate reductase (MDAR4), glutathione peroxidase 5 (GPX5) and glutathione peroxidase 6 (GPX6). The downregulation of genes involved in ascorbate

and glutathione cycles are in agreement with the finding of significantly reduced antioxidant content, measured by the FRAP assay, in basil leaves subjected to chilling stress compared to those treated in ambient conditions. Downregulation of antioxidative genes and the reduced accumulation of antioxidants in response to chilling stress suggests that basil plants were compromised in their ability to defend themselves against oxidative stress during chilling stress. There is a possibility that this reduced defence against oxidative damage may have allowed for increased production of ROS to occur despite its negative effect on the intactness of cellular components under chilling stress leading to damage to cellular membranes, chloroplasts, proteins and other cellular components. There were various chaperone proteins such as HSP70, HSP81, 20 kDa chaperonin and a cyclophilin-like peptidyl-prolyl cis-trans isomerase that were upregulated in basil in response to chilling conditions, which suggests proteins may have possibly undergone misfolding, triggering the induction of protective chaperones (via the unfolded protein response) to restore native folding of misfolded proteins. This misfolding of proteins could possibly occur in response to chilling conditions or due to adverse reaction with ROS which is sometimes referred to as oxidative protein folding.

Lipid peroxidation increased after exposure to chilling temperatures and also occurs in response to increased oxidative stress which contributes to increased permeability to ions by membranes. The metabolic pathways of lipid metabolism revealed downregulation of genes involved in lipid synthesis (phosphatidylglycerolphosphate synthase 2 (PGPS2) and monogalactosyl diacylglycerol synthase 1 (MGD1)) and modification (FAD2, FAD5, FAD6, FAD7 and FAD8). These changes could possibly increase the membranes' susceptibility to damage by chilling conditions through processes such as membrane rigidification. In addition, reduced lipid synthesis could limit the replacement of damaged lipid components. Hence, it may be possible that

these molecular changes that occurred in response to chilling conditions could be responsible for the damage experienced by the basil cell membrane under chilling conditions.

Increased levels of ROS may cause disruption of the chloroplast components including PSII and the thylakoid membrane. Among downregulated genes in response to chilling stress were those associated with the enriched biological process of chlorophyll biosynthesis and included genes such as chlorophyllide an oxygenase, prophobilinogen deaminase, a probable 1-dexoy-D-xylulose-5-phosphate synthase and tetrapyrrole-binding protein. Any reduction in chlorophyll molecules could reduce the antenna size of the PSII and could be of adaptive value during chilling stress so that less light is absorbed by antenna pigments; thus, balancing the reduced utilisation by the Calvin cycle of energy generated by the light reactions of photosynthesis and, thereby, reducing the production of ROS via chilling-enhanced photo-oxidation. However, reduced chlorophyll biosynthesis during chilling stress could reduce photosynthesis and thereby the growth of the plant compared to plants treated at ambient conditions but for the present project this was not an issue as plants would be full grown (ready for marketing) when potentially exposed to chilling temperatures during transport. The downregulation of chlorophyll biosynthetic genes would mean less absorption of light and thereby contribute to the reduced delayed chlorophyll fluorescence that was observed when basil was exposed to chilling temperatures.

The transcriptomic reprogramming in response to chilling conditions showed that chilling conditions have a widespread impact on basil plants with metabolism appearing to be arrested. The changes occurring in response to chilling conditions were compatible with the findings from the physiological experiments and show that chilling temperature causes damage to cellular components and results in suppression of plant metabolism. Hence, in order to induce chilling tolerance in chilling sensitive

basil, a treatment was required that could have a global effect and cause suitable transcriptomic reprogramming in plant. The irradiation of light enriched in FR light is known to positively regulate freezing tolerance in *A. thaliana*. The study by Franklin and Whitelam (2007) showed that irradiation of *A. thaliana* with LR:FR light from ZT4-ZT8 during short days could induce freezing tolerance in *A. thaliana* without prior acclimation of the plant at lower temperatures. In the present study, it was proposed that such a treatment might, similarly, induce chilling tolerance in a chilling sensitive plant. Basil was therefore irradiated with supplementary FR, i.e. LR:FR, light from ZT4-ZT8 on each of the four days of the treatment and assessed for the efficacy of the treatment in inducing chilling tolerance in chilling sensitive basil. Physiological assays showed that pre-treatment of basil with LR:FR light for four days between the period of ZT4-ZT8 significantly reduced chilling induced damage. Thus, treated plants showed reduced electrolyte leakage, lower accumulation of ROS and greater chlorophyll fluorescence yield chilling stress when pre-treated with LR:FR light compared to plants maintained in standard HR:FR light. Through analysis of transcriptomic profile changes in response to chilling temperatures those genes that showed either "permanent" upregulation or "temporary" upregulation in response to LR:FR light showed enrichment of biological process that were all related to plant responses to oxidative stress. These processes included, cell redox homeostasis, cellular homeostatic processes and oxidation reduction. Highly upregulated genes in these categories included glutaredoxin-C1, monothiol glutaredoxin-S2, peroxiredoxin-2F and cytochrome P450 90C. In addition, genes showing temporary upregulation in response to LR:FR light conditions at ambient temperature prior to the plants exposure to chilling conditions also showed enrichment of biological processes such as response to cadmium ion, response to metal ion and response to inorganic substance and included genes such as aconitate hydratase 1, aconitate hydratase 2, catalase 2, catalase 3 and peroxidase 42. These genes encode proteins that play roles in detoxification of ROS

content in leaves as well as in limiting the further generation of oxidative stress in the cell. These processes were also found enriched among upregulated genes in basil plants exposed to 24h of chilling stress but pre-treated with LR:FR light compared to basil plants maintained in standard HR:FR light. Processes such as response to oxidative stress, response to abiotic stimulus, response to metal ion, response to cadmium ion, response to osmotic stress, response to salt stress, oxidation-reduction were also found enriched among genes expressed at higher levels following exposure to chilling conditions in plants that were pre-treated with LR:FR light compared to basil plants maintained in standard HR:FR light.

It was also noticeable that genes related to aquaporins were, according to analysis of RNAseq data, upregulated in plants treated with LR:FR. Aquaporins play a role in maintaining cellular water homeostasis at times of abiotic stress. In the present study, these genes were downregulated during chilling stress but upregulated in plants treated with LR:FR at ambient conditions. Investigation of the regulation of water relations in basil pre-treated with or without LR:FR showed that increased aquaporin expression at ambient conditions before exposure to chilling conditions can allow basil cells treated with LR:FR light to have possibly greater water content and greater abundance of aquaporins. This could in turn, allow for basil plants pre-treated with LR:FR light to show greater tolerance to water imbalance stress posed by chilling temperatures compared to basil treated with HR:FR light. Thus, aquaporins could possibly, in part, contributing to the increased chilling tolerance in otherwise chilling sensitive basil.

The physiological and molecular changes occurring in the basil plants in response to LR:FR light have revealed that the modified light regime is effective in reducing chilling damage in chilling sensitive basil and has provided possible suggestions that may account for basil's differential responses to chilling temperatures when pre-treated

with or without LR:FR light. The use of such a modified growing environment during the final four days of growth before packing and transporting pot-grown basil could provide a commercially useful treatment that would enable basil, and other herbs that are naturally chilling tolerant, to be transported at lower temperatures and thereby reduce the metabolic rate and lead to an extension of the shelf life of these plants which in itself could lead to less wastage.

7.1 Future Work:

In this project, the irradiation of LR:FR light for four days was proven to be effective in inducing chilling tolerance in chilling sensitive basil. Therefore, it would be of interest to investigate the effect of different number of days of LR:FR light irradiation on basil's chilling tolerance. In order to do this, basil can be either maintained in HR:FR light or treated with periodic LR:FR light (ZT4-8) for 2 d, 4 d and 6 d before being stored in chilling or ambient conditions for 24 h. Chilling tolerance of basil can be assessed using physiological assays including electrolyte leakage assay, lipid peroxidation and ROS accumulation. The differences in the measurements should indicate the shortest duration of LR:FR light irradiation required to induce chilling tolerance in basil. The findings can also be used to identify if irradiation of basil for more than four days induces a greater chilling tolerant behaviour than that observed in four days.

Chilling tolerance of basil in this present project was assessed immediately after 24 h of chilling stress. Therefore, the lasting effect of LR:FR light induced chilling tolerance in basil is not known, and can be followed as part of future work. In order to investigate this, basil can be maintained in control or periodic LR:FR light conditions for four days and subsequently stored at chilling temperatures for 24 h. Basil can then be transferred and kept at ambient conditions for 0 d, 2 d and 4 d to follow the LR:FR light induced chilling tolerant effect. Basil can be harvested on 0 d, 2 d and 4 d after 24 h of

chilling stress and used to carry out physiological assays on. The results can be used to identify if the effect of LR:FR light induced chilling tolerance declines over time. If so, then it may also show the time taken by LR:FR light to reach the same level of electrolyte leakage, ROS accumulation and lipid peroxidation as basil maintained in HR:FR light.

Enriched GO terms from genes found temporarily upregulated in basil by LR:FR light were involved in ABA and JA synthesis. Upregulation of genes involved in ABA and JA synthesis indicates that their signalling was probably increased and may have contributed to basil increased tolerance to chilling conditions. Thus, it would be of interest to create RNAi lines of ABA or JA and reduce their expression in basil plants and assess whether their reduced contents impact on basil's response to chilling temperatures. RNAi lines of ABA or JA can also be treated with LR:FR light to assess the impact of ABA or JA reduced expression on LR:FR light mediated effect of chilling tolerance on basil plants. This can elucidate or confirm the involvement of ABA and JA induction by LR:FR light in enhancing chilling tolerance in sensitive basil. Basil is evidently not a genetic model plant, and RNAi silencing technique has not been frequently utilised. However, it is expected that the latest genome editing techniques may make such experiments realistic in the not too distant future.

The gene ontology analysis of differentially expressed genes from the three RNA sequencing experiment have suggested that genes linked to redox homeostasis, particularly those involved in the synthesis of polyamines to play important role in basil chilling tolerance. Hence, it would be of interest to investigate the role of arginine decarboxylase 1 and arginine decarboxylase 2 in basil's response to chilling temperatures. Arginine decarboxylase 1 and arginine decarboxylase 2 are involved in the synthesis of polyamines and found upregulated in basil's response to cold temperatures (according to our first RNA sequencing experiment) as well as in basil

stored at chilling temperatures after pre-treatment with LR:FR light (third RNA sequencing experiment). Transgenic basil plants containing gain-of-function forms of mutation of ADC1 or ADC2 can be created to study the role of ADC1 or ADC2 overexpression in basil cells during chilling stress. The wild type basil and transgenic basil plants can be subjected to chilling stress for 24 h and the difference in accumulation of polyamines, accumulation of ROS and electrolyte leakage measured to study the impact of ADC1 or ADC2 role in basil's response to chilling temperatures.

8 APPENDIX

All supplementary material is attached on the external media.

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