Evaluating Role of Phosphatidic Acid in Cold Stress Tolerance in Silage Corn

By

© Jiaxu Wu

A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of

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Approved:

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Abstract

Extreme cold weather conditions and short growing seasons during early seedling establishment are the major causes of the low forage yield of silage corn (*Zea mays* L.). The experiments were conducted under controlled environmental conditions to determine the effects of different temperature regimes (25°C, 20°C, 15°C, 10°C and 5°C) on morphological, physiological and biochemical attributes of two silage corn genotypes (Yukon-R, A4177G3-RIB) during the early growth stage. Results indicated that cold stress significantly affected seedling growth, photosynthesis, reactive oxygen species (ROS) accumulation and antioxidant enzyme activities. The chlorophyll content, photosynthetic rate, and maximal photochemical efficiency of photosystem-II were drastically decreased under cold conditions. Besides, cold stress induced the accumulation of hydrogen peroxide and malonaldehyde contents. Increased proline content and enzymatic antioxidants were found to alleviate oxidative damage under cold stress. Yukon-R showed significantly higher proline content, and enzymatic antioxidant activities than A4177G3-RIB when the temperature was lower than 10°C. Furthermore, the role of phospholipids, particularly phosphatidic acid (PA) in cold tolerance was investigated. Lipidomic results showed that the membrane lipids phosphatidylcholine and phosphatidylglycerol levels were decreased in contrast to increased levels of phosphatidylethanolamine and PA under cold temperatures. Specifically, Yukon-R showed significantly higher PA, phosphatidylcholine and phosphatidylglycerol contents than A4177G3-RIB in both leaf and root membranes under cold conditions, suggesting their positive roles (stress signaling transduction, maintaining cellular integrity and photosynthetic processes, respectively) in cold tolerance. Taken together, the study suggests that: (i) Yukon-R could be considered a potential candidate genotype to be grown in the

boreal climate; (ii) phospholipid remodeling and PA accumulation in leaf and root are required for silage corn cold stress acclimation at the early growth stage.

General Summary

Cold stress is one of the major abiotic factors that negatively affect the growth and productivity of crops in the boreal agroecosystem. Silage corn is an important forage crop in Newfoundland and Labrador (NL), Canada. Specifically, silage corn seedling establishment and subsequent productivity are strongly affected by low temperatures during the early growth stage. To explore this issue, growth chamber experiments were conducted to investigate the effects of cold stress on morphological, physiological and biochemical attributes in two silage corn genotypes (Yukon-R, A4177G3-RIB). Our results also showed that Yukon-R could be considered a potential candidate genotype to be grown in the boreal climate. In addition, lipidomic analysis was employed to understand alterations in phospholipid metabolism in response to cold temperatures. Similar phospholipid remodelling trends were found in both genotypes and tissues (leaf and root). However, Yukon-R showed higher phosphatidic acid (PA) contents in leaf and root membranes than A4177G3-RIB under cold stress, which might be responsible for the superior cold tolerance observed in Yukon-R.

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Co-authorship statement

Chapters of this thesis are based on different manuscripts. The list of the manuscripts (in preparation or submitted) and contributions made by authors are shown as follow:

Chapter 1: Introduction and Literature Review is modified from the manuscript entitled "Effects of cold stress on plant growth and the functions of phosphatidic acid in plant cold stress tolerance". Revised version of this paper is submitted to ***Environmental and Experimental Botany*** (*Under review*).

This manuscript is co-authored by Jiaxu Wu, Muhammed Nadeem, Lakshman Galagedara, Raymond Thomas and Mumtaz Cheema. The primary author, Jiaxu Wu, wrote the first draft. Drs. Raymond Thomas and Lakshman Galagedara provided critical comments. Dr. Muhammad Nadeem and Dr. Mumtaz Cheema conceived the concept.

Chapter 2: Effects of chilling stress on morphological, physiological and biochemical attributes of silage corn genotypes during seedling establishment. This paper has been published in ***Plants*** (https://doi.org/10.3390/plants11091217).

This manuscript is co-authored by Jiaxu Wu, Muhammed Nadeem, Lakshman Galagedara, Raymond Thomas and Mumtaz Cheema. Jiaxu Wu performed most of the experiments and wrote the first draft. Dr. Muhammed Nadeem helped in experimental design, data analysis and reviewing the first draft. Drs. Raymond Thomas and Lakshman Galagedara provided critical suggestions in data analysis, reviewed and edited the manuscript. Dr. Mumtaz Cheema supervised this study, helped in data analyses, provided critical review and edited the manuscript.

Chapter 3: Lipidomics remodelling: a strategy to enhance cold stress tolerance in silage corn will be submitted to ***Scientific Reports*** (*internal review in process*).

This manuscript is co-authored by Jiaxu Wu, Muhammed Nadeem, Raymond Thomas, Lakshman Galagedara and Mumtaz Cheema. For this manuscript, Jiaxu Wu conducted most of the experiments and wrote the first draft. Dr. Muhammed Nadeem was involved in experimental design, data analysis and reviewing the manuscript. Drs. Raymond Thomas and Lakshman Galagedara helped to finalize the methodology, provided critical suggestions in experiment design and data analysis, and reviewed and edited the manuscript. Dr. Mumtaz Cheema supervised overall this study and edited the manuscript.

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List of Abbreviations and Symbols

$^1O_2$ – Singlet oxygen

ABI1 – Abscisic acid intensive 1

ACBP1 – Acyl-CoA binding protein 1

ANOVA – Analysis of variance

AOX – Alternative oxidase

APX – Ascorbate peroxidase

AsA – Ascorbic acid

Ca^{2+} - Calcium ion

CAT – Catalase

CBF/DREB1 – C-repeat binding factor/dehydration-responsive element-binding protein 1

Chl *a* – Chlorophyll a

Chl *b* – Chlorophyll b

COR genes – *Cold-responsive* genes

DAG – Diacylglycerol

DGDG – Digalactosyldiacylglycerol

DGPP – Diacylglycerol pyrophosphate

DGK – Diacylglycerol kinase

DHAR – Dehydroascorbate reductase

EDTA – Ethylenediaminetetraacetic acid

FAD – Fatty acid desaturates

Fv/*Fm* – Maximum quantum yield of photosystem II

GB – Glycine betaine

Gro3*P* – Glycerpiol-3-phosphate

GPX – Glutamine peroxidase

GR – Glutathione reductase

GSH – Glutamine

H_2O_2 – Hydrogen peroxide

HSPs – Heat shock proteins

IBPs – Ice-binding proteins

KI – Potassium iodide

LEA proteins – Late embryogenesis abundant proteins

LPA – Lysophosphatidic acid

LPC – Lysophosphatidylcholine

LPE – Lysophosphatidylethanolamine

LPG – Lysophosphatidylglycerol

MDA – Malondialdehyde

MDHAR – Monodehydroascorbate

MGDG – Monogalactosyldiacylglycerol

MPK – Mitogen-activated protein kinase

NADP – Nicotinamide adenine dinucleotide phosphate

NBT – Nitro blue tetrazolium

NL – Newfoundland and Labrador

NO - Nitric acid

NPC – Non-specific phospholipase C

$O_2^{\bullet-}$ - Superoxide

OH• - Hydroxyl radical

PA – Phosphatidic acid

PABD – PA binding domains

PAK – Phosphatidic acid kinase

PAP - Phosphatidic acid phosphatase

PBS – Phosphate-buffered saline

PC – Phosphatidylcholine

PCA – Principal Component Analysis

PE – Phosphatidylethanolamine

PG – Phosphatidylglycerol

PI – Phosphatidylinositol

PI-PLC – Phosphoinositide phospholipase-C

PI4KIII – Type-III phosphatidylinositol-4-kinases

PI4P – Phosphatidylinositol-4-phosphate

PI4P5K – Phosphatidylinositol-4-phosphate 5-kinase

PIP_2 – Phosphatidylinositol 4,5-bisphosphate

PLA – Phospholipase-A

PLC – Phospholipase-C

PLD – Phospholipase-D

PPDK – Pyruvate phosphate dikinase

PPFD – Photosynthetic photon flux density

PS – Phosphatidylserine

PS-II – Photosystem-II

PVP – Polyvinylpyrrolidone

q_p – non-photochemical quenching coefficient

qRT-PCR – Real time quantitative reverse transcription polymerase chain reaction

R^2 – Correlation coefficient

ROS – Reactive oxygen species

RNA – Ribonucleic acid

Rubisco – 1,5-*bis*phosphate carboxylase/oxygenase

SOD – Superoxide dismutase

TBA – Thiobarbituric acid

TCA – Trichloroacetic acid

Chapter 1

Introduction and Literature Review

1.1.Introduction

Silage corn (*Zea mays* L.) has emerged as an important forage crop for the dairy and livestock industry in Newfoundland and Labrador (NL), Canada. NL dairy and livestock industries face challenges of insufficient silage, forage, or grain production, and they have to depend on substantial imports from other provinces which put a heavy economic and environmental burden due to high feed and shipping prices, and substantially increased carbon footprints due to transport. These challenges can be reduced by growing local silage corn or forages that might bridge the gap between production and consumption. As a tropical plant, silage corn is very sensitive to cold temperatures, especially during the early transition stages from heterotrophic to autotrophic growth (Andrews et al. 1995; Leipner and Stamp 2009). However, the low temperatures in the early growing season restricts seedling establishment and hence limits the forage production in NL (Nadeem et al. 2019a; Spaner et al. 2000). It is reported that the average temperatures in NL during the growing season (June – October) are between 10 °C - 12 °C, which are considered cold stress for corn growth (Nadeem et al. 2019b). Therefore, plastic mulching is a common practice to enhance the crop heat units during germination and early seedling stage (Kwabiah 2003).

Cold stress has multiple effects on corn growth from morphological to molecular aspects. Several studies indicated that Cold stress increases seed germination time, reduces seedling emergence and seedling vigor under low growth temperatures (Farooq et al. 2009; Hussain et al. 2018; Ji et al. 2017; Yadav 2010). Landi and Crosbie (1982) demonstrated that vegetative growth of corn seedlings was reduced by more than 50% by low temperatures (8 °C/3 °C and 10 °C/5 °C,

day/night) compared to control temperature (16 °C/10 °C, day/night). In addition, Rymen et al. (2007) reported that cold stress reduced the leaf growth and the total number of corn leaves through reduced cell division and cell elongation. cold stress also strongly affects corn photosynthetic performance, decreased CO_2 acclimation rate, chlorophyll content, and the efficiency of photosystem-II are all observed, which can further contribute to yield loss (Foyer et al. 2002; Holá et al. 2007). In the present study, the main aim is to determine the effects of cold stress on physiological, biochemical and membrane lipidomic profiles to enhance the understanding of the role of lipid metabolism at the early growing stage in cold stress adaptation of silage corn (Kwabiah et al. 2003; Zaeem et al. 2019). The hypothesis is that cold stress would affect the seedling growth, morphological, physiological and biochemical attributes of the two silage corn genotypes (Yukon-R and A4177G3-RIB), which are selected based on their agronomic performance under the field condition in NL (Nadeem et al. 2019b).

This study aims: 1) to determine the effects of cold and non-cold stress on seedling growth, morphological, physiological, and biochemical attributes of silage corn at early growth stage, 2) to investigate the role of phospholipidome mediation in cold stress tolerance in silage corn at the early growing stage.

1.2. Literature review

1.2.1. Abiotic stress

The rapid increase in population and uncertain and erratic weather pattern has posed a significant threat to global food security (Ehrlich and Harte 2015; Lesk et al. 2016). To meet the ever-raising food and feed demands of the human and livestock industry in the mid-21st century, it is inevitable to increase 60-70 % of global food production from current levels (Bailey-Serres et al. 2019;

Mittler 2006; Zhu 2016). However, abiotic stresses, such as thermal (cold and heat), drought, soil acidity, salinity, nutrient deficiencies, and heavy metal toxicities cause significant yield reduction of major crops and hence may pose a serious threat to global food security (Zhang et al. 2018).

1.2.2. Effects of cold stress on crop plants

Cold stress is responsible for serious economic and food production losses, which is considered one of the major environmental factors limiting crop production due to geographical distribution and the increasing frequency of extremely low temperature events (Ding et al. 2019; Shi et al. 2018). It is reported that over 60% of the earth's land area suffers from an average minimum temperature below 0 °C (Rihan et al. 2017; Źróbek-Sokolnik 2012). Severe freezing during the early and late growing season is a threat to many crops in the northern region of America, Europe, and Asia (Gao 2016; Gobin 2018; Juurakko et al. 2021; Shannon and Motha 2015). For instance, an unusual forest event in September 2018 destroyed $4 billion worth of crops in Alberta, Canada (Environment Canada 2021). Similarly, in January 2008, a large-scale snowstorm in China significantly affected nearly 40% of the winter crops in China (Zhou et al. 2011). Depending on the different adverse effects on plant growth, cold stress could be divided into cold stress (temperatures lower than 20 °C but higher than 0 °C) and freezing stress (temperatures lower than 0 °C) (Chinnusamy et al. 2007; Guo et al. 2018; Liu et al. 2018; Wang et al. 2016a). Chilling stress influences a wide range of morphological, physiological and biochemical processes in growing plants, for instance, reduced plant growth and development, photosynthesis and sugar translocation, membrane fluidity and metabolic homeostasis (Mukhopadhyay and Roychoudhury 2018). However, the effects of chilling stress may differ between temperate and tropical plants (Ding et al. 2019). For instance, the crops from semitropical and tropical regions are classified as chilling-

sensitive, including corn (Foyer et al. 2002; Leipner and Stamp 2009), tomato (Park et al. 2006), rice (Martynenko et al. 2015), soybeans (Bramlage et al. 1978) and cotton (Bolger et al. 1992). On the other hand, plants from temperate regions are able to tolerate chilling stress and survive in freezing temperatures, such as winter wheat, spinach, canola and *Arabidopsis* (Ruelland et al. 2009).

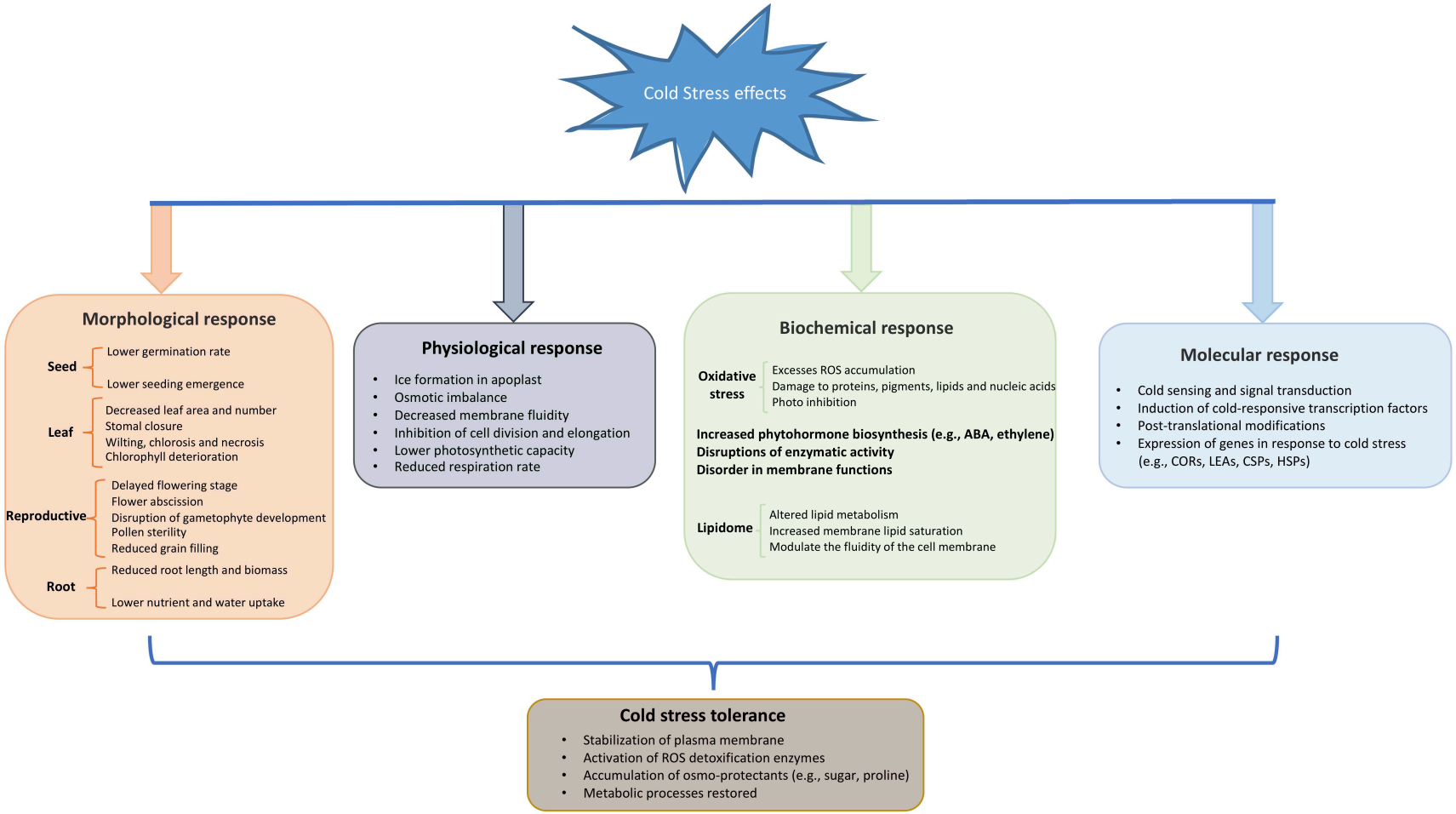
Cold Stress effects
Morphological response
Seed
Lower germination rate
Lower seeding emergence
Leaf
Decreased leaf area and number
Stomal closure
Wilting, chlorosis and necrosis
Chlorophyll deterioration
Reproductive
Delayed flowering stage
Flower abscission
Disruption of gametophyte development
Pollen sterility
Reduced grain filling
Root
Reduced root length and biomass
Lower nutrient and water uptake
Physiological response
Ice formation in apoplast
Osmotic imbalance
Decreased membrane fluidity
Inhibition of cell division and elongation
Lower photosynthetic capacity
Reduced respiration rate
Biochemical response
Oxidative stress
Excesses ROS accumulation
Damage to proteins, pigments, lipids and nucleic acids
Photo inhibition
Increased phytohormone biosynthesis (e.g., ABA, ethylene)
Disruptions of enzymatic activity
Disorder in membrane functions
Lipidome
Altered lipid metabolism
Increased membrane lipid saturation
Modulate the fluidity of the cell membrane
Molecular response
Cold sensing and signal transduction
Induction of cold-responsive transcription factors
Post-translational modifications
Expression of genes in response to cold stress (e.g., CORs, LEAs, CSPs, HSPs)
Cold stress tolerance
Stabilization of plasma membrane
Activation of ROS detoxification enzymes
Accumulation of osmo-protectants (e.g., sugar, proline)
Metabolic processes restored

Figure 1.1 Effects of cold stress and cold tolerance mechanisms of growing plants. ABA: abscisic acid; APX: ascorbate peroxidase; AsA: ascorbic acid; CAT: catalase; CORs: cold-regulated genes; CSPs: cold shock proteins; HSPs: heat shock proteins; GPX: glutathione peroxidase; GSH: glutathione; LEAs: late embryogenesis abundant proteins; Put: putrescine; ROS: reactive oxygen species; SOD: superoxide dismutase; Spd: spermidine; Spm: spermine.

1.2.3. Plant morphological, physiological, biochemical and molecular responses under cold stress

Plants face cold stress from very early growth stages, which causes impaired germination, seedling emergence, overall plant growth, development, and biomass production (Figure 1.1). Researchers demonstrated that increased seed germination time, reduced seedling emergence and seedling vigor under low growth temperatures (Farooq et al. 2009; Hassan et al. 2021; Hussain et al. 2018). After seed germination and seedling emergence, the leaf initiation rate depends on growth temperatures (Hussain et al. 2018). Yadav (2010) also observed severe leaf wilting, chlorosis and necrosis in rice plants exposed to a freezing temperature of -2 °C for 12 h. Similarly, Sun et al., (2017) observed a reduction in root architecture and disorder in root tip cells in sugarcane plants when grown at 4 °C for three days. The reduction in root architectural growth results in reduced water, nutrient uptake and nutrient use efficiency (Grossnickle 2005). Ercoli et al. (2004) demonstrated a decreased nitrogen (N) uptake in sorghum plants under cold temperatures (2 °C, 5 °C and 8 °C), which resulted in reduced plant height and nitrogen concentrations in plant tissues. In addition, the reproductive stage is very prone to cold stress (Hassan et al. 2021). The yield production of some cold-sensitive crop species, such as corn and rice, is severely affected in temperate regions (Arshad et al. 2017). The early pollen microspore stage and anthesis are considered the most sensitive stages to cold injury for rice (Mitchell et al. 2016). Ji et al. (2017) reported that wheat grain yield decreased significantly with decreasing temperature. Generally, cold temperatures delay the flowering, disturb gametophyte and pollen development during reproductive growth, these effects will contribute to infertility at the seed set (Thakur et al. 2010; Zinn et al. 2010). Furthermore, evidence suggests that during the anthesis stage, cold conditions such as 17 °C have a more negative impact on pollen function, anther size and spikelet fertility in

cold-sensitive rice cultivars compared to the tolerant ones (Dingkuhn et al. 2015; Zeng et al. 2017). Poor grain development is mainly due to restricted carbohydrate supply, and studies support the fact that cold stress influences vital enzymes activities such as sucrose synthase (SUS) and sucrose phosphate synthase (SPS), which are involved in carbohydrate metabolism during the grain-filling phase (Arshad et al. 2017; Bilska-Kos et al. 2020; Savitch et al. 2000). Therefore, low grain yield under suboptimal temperature (about 15 °C) is associated with the reduced allocation of resources to grain resulting in poor seed filling and seed development (Zinn et al. 2010). It is obvious from the above discussion that cold stress has negative effects on cell division, cell elongation, and enzyme activities at seed germination, seedling emergence, vegetative and reproductive growth stages resulting in reduced final biomass production.

Plant cell elongation and division are inhibited at cold temperatures, which play critical roles in plant growth and development. The study showed that the root mitotic index was also restricted in rice when the temperature was lower than 10 °C (Chen et al. 2011). In addition, cold stress affects several physiological processes in growing plants, such as photosynthetic activities and mitochondrial respiration (Figure 1.1) (Hassan et al. 2021; Kerbler et al. 2019; Yadav 2010). The chlorophyll contents were decreased by cold stress, which might be because of the inhibition of chlorophyll biosynthetic enzymes (Zhao et al. 2020). Li et al. (2021) found significant damage to epidermis cells and sponge, and reduced chlorophyll in tobacco leaves when grown at low temperature. Moreover, cold stress causes dysfunction of all major photosynthesis components such as stomatal conductance, electron transport and carbohydrate metabolism (Allen and Ort 2001; Long and Spence 2013). Generally, cold-induced chloroplast ultrastructural symptoms include chloroplast swelling, smaller size and number of starch granules, thylakoid swelling and distortion resulting in an impairment in the photosynthetic machinery (Kratsch and Wise 2000). Furthermore,

cold stress decreases light absorption in the thylakoid electron transport of photosystem II (PS-II) and increases excitation energy quenching in the light-harvesting antennae (Bilska and Sowiński 2010). Allen and Ort (2001) and Andrews et al. (1995) demonstrated decreased leaf maximum photochemical efficiency of PS-II (*Fv/Fm*), the efficiency of excitation energy transfer to open PS-II reaction center (*Fv'/Fm'*), and non-photochemical quenching coefficient (q_p) values indicating photoinhibition of PS-II in growing plants under cold stress. Excess photons, moreover, may lead to photooxidative destruction of the photosynthesis apparatus (Aroca et al. 2001). Suboptimal temperature conditions can also lower the activities and contents of several photosynthesis enzymes, including ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), pyruvate phosphate dikinase (PPDK) and nicotinamide adenine dinucleotide phosphate (NADP) malate dehydrogenase in several tropical (C_4) plants (Doubnerová and Ryšlavá 2011; Kingston-Smith et al. 1997; Naidu et al. 2003; Salesse-Smith et al. 2020). This information suggests that along with PS-II, all these factors reduced more than 60% of the carbon assimilation capacity in growing plants under cold stress conditions (Long and Spence 2013).

Plant respiration provides energy for cellular maintenance, active transport, and biosynthesis, which ability is also significantly reduced by cold temperatures (Turk et al. 2014). Respiration rate and Adenosine triphosphate (ATP) biosynthesis rate were decreased by 78% and 77%, respectively, at 4 °C compared to 25 °C (Kerbler et al. 2019). Wang et al. (2016b) reported impaired starch breakdown and respiration metabolism causing a 40% low emergence rate in rice seed under moderate cold field conditions. Atkin and Tjoelker (2003) demonstrated that at low temperatures, the mitochondria respiratory flux is predominantly limited by catalytic enzyme activity.

It is known that cold stress can reduce the fluid state of cellular membranes and increase rigidity, which changes in physical conformation play a negative role in stabilizing membrane lipids and proteins (Barrero-Sicilia et al. 2017; Ding et al. 2019). Early frost injury during late spring and early fall frosts are more severe than chilling stress, which promotes ice formation in the apoplast and disturbs the cellular membrane functions *via* decreasing water potential and causes freezing-induced cell dehydration and osmotic shrinkage (Ding et al. 2019; Long and Spence 2013; Pearce 2001). Eventually, ice can penetrate and spread into the cell, causing intercellular structure deterioration and tissue death. Extracellular ice formation imposes dehydration stress on plant cells, and osmotic contraction is associated with the endocytosis vesicle formation (Mukhopadhyay and Roychoudhury 2018). Plant cells experience osmotic contraction during the thaw, which is accompanied by surface area expansion. However, when these surface areas are too large to recover, protoplast damage occurs, causing expansion-induced lysis (Ruelland et al. 2009). Besides, freezing stress also forms hexagonal II phase or interlamellar attachments when plasma membranes are in proximity to the endomembranous. Both will lead to loss of osmotic expansion during the thaw. Moreover, interlamellar attachment accumulation can cause fracture-jump lesions in plants.

The plasma membrane is considered a primary target and sensitive biosensor of cold stress (Lamers et al. 2020; Penfield 2008). Exposure is known to cause rigidification and impairment in the normal membrane functions, such as ion exchange and electron transfer reactions as shown in Figure 1.1. Cold stress contributes to the over-accumulation of reactive oxygen species (ROS) in growing plants (Figure 1.2). Generally, hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$) and hydroxyl radical (OH•) are produced under cold stress and their production is linearly correlated with the stress intensity (Hassan et al. 2021). The over-accumulation of H_2O_2,

$O_2^{\bullet-}$, $^1O_2$ and OH• leads to oxidative stress, which impairs various cellular functions by causing peroxidation of the membrane, photosynthesis impairment, damaging nucleic acids, oxidizing proteins and lipids (Castro et al. 2021; Chinnusamy et al. 2007; Gill and Tuteja 2010). It has been found that lipid peroxidation increases membrane leakage and decreases membrane fluidity, which is also considered the main reason for damaged membrane-localized proteins associated with ion-channels, receptors, and enzyme architecture (Das and Roychoudhury 2014).

Cold stress affects the stability and solubility of proteins which disrupt the metabolic reactions for plant growth and development (Siddiqui and Cavicchioli 2006). The citric acid and Calvin cycles are central biochemical pathways for generating cellular energy and organic compounds necessary for plant metabolic processes, respectively (Sharma et al. 2020; Sweetlove et al. 2010). Proteomic analysis showed that some vital enzymes of these two cycles (malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and transketolase) were down-regulated remarkably in spring wheat upon prolonged cold temperature exposure (4 °C for 21 and 42 days) (Rinalducci et al. 2011). Dong et al. (2020) demonstrated that there 14 genes involved in the citrate cycle pathway, were more highly expressed in Kentucky bluegrass cold-tolerant varieties than normal varieties after 3-days of 0 °C treatment. The antioxidant system can alleviate oxidative stress under cold stress in a short time. However, activities of ROS scavenging enzymes including superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxide (APX) may be reduced after prolonged exposure or high intensity of cold stress (Herath 2018). Moreover, cold stress affects RNA folding process, which disturbs the translation efficiency by generating secondary structures in RNA molecules, and these misfolded structures will disrupt cellular normal transcription functions in response to cold stress (Kang et al. 2013).

Phytohormone metabolism is also involved in plant biochemical response during cold stress. An increase in abscisic acid (ABA) levels was found in many plant species, and exogenous application of ABA increases plant cold stress tolerance (Baron et al. 2012; Heidarvand and Maali Amiri 2010; Huang et al. 2017; Maruyama et al. 2014). Similarly, cold stress promotes ethylene, salicylic acid and jasmonic acid biosynthesis in plants (Catalá et al. 2014; Dong et al. 2014; Hu et al. 2013; Jung et al. 2016). Some hormone bioactivity and biosynthesis such as gibberellin and cytokinin were suppressed during cold stress (Eremina et al. 2016).

Growing plants can sense any external stimuli and signal transduction which play a significant role under different environmental conditions. Therefore, the success of plant cold perception is crucial for initiating downstream signaling and sustaining cold stress response. It is reported that the changes in plasma membrane fluidity, calcium ion (Ca^{2+}) channels and phytochrome are responsible for the detection of cold temperatures (Ding et al. 2019).

Plants adapt to different kinds of messenger molecules such as Ca^{2+}, ROS biosynthesis and PA for signal transduction under cold stress. Ca^{2+} is essential in signal transduction and plays a vital role in plant thermal perception (Figure 1.2), Ca^{2+} sensors such as calmodulins (CaMs), Ca^{2+}-dependent protein kinases (CDPKs) and CaM-like proteins (CMLs) play a critical role in cold triggered Ca^{2+} signaling pathway (Yuan et al. 2018). Such downstream signaling events include regulating gene expression, phosphorylation and ROS production, helping growing plants in cold stress adaption (Reddy et al. 2011; Yuan et al. 2018).

Reactive oxygen species (ROS) also play a vital role in plant cold stress response (Baxter et al. 2013; Chinnusamy et al. 2007). Frostbite1 (*FRO1*) is localized in mitochondria, which encodes the Fe-S subunit of complex I (NADH dehydrogenase) in the mitochondria electron transfer chain

(Lee et al. 2002). The *FRO1* mutants impaired cold-responsive gene expression and showed constitutive accumulation of H_2O_2 and O_2^- in the leaves, suggesting that ROS may affect nuclear gene expression under cold stress by serving as metabolic signal messengers (Lee et al. 2002).

Phosphatidic acid (PA) acts as a key second messenger in regulating a plant's response to abiotic stress. Cold stress results in an increase in PA levels, and many lipidomic studies document increased levels of PA during chilling and freezing stress in various plant species (Gu et al. 2017; Welti et al. 2002; Zhao et al. 2021; Zheng et al. 2016). PA has been reported to interact with proteins to modulate catalytic activities and intercellular distribution (Hong et al. 2016). Besides, researchers have indicated a role for PA in improved crop agronomic performance as well as a potential application as a biomarker for distinguishing different cold tolerant silage corn genotypes when grown under cold growth conditions in boreal climate (Nadeem et al. 2019b; Nadeem et al. 2020).

The C-repeat binding factor/dehydration-responsive element binding protein 1 (CBF/DREB1) - dependent signal pathway was identified and showed a complex regulatory cold response network, which involves transcriptional, post-transcriptional and post-translational processes in *Arabidopsis* (Fowler and Thomashow 2002). Generally, the *CBF* genes are rapidly induced under low temperatures in less than 15 min and reach their maximum expression level after 1 to 2 h. The *CBF* genes could encode APETALA2/ethylene-responsive element binding factor (AP2/ERF)-type transcriptional factors that can specifically bind to the conversed CRT/DRE motifs (A/GCCGAC) in the promoter of *COR* genes and then induce their expression (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Stockinger et al. 1997).

The functions of *CBF* genes have been recognized in many plant species, and the transgenic approach shows that induction of *COR* gene expression enhances plant cold stress tolerance (Ito et al. 2006; Qin et al. 2004; Savitch et al. 2005). Inducer of *CBF* expression 1 (ICE1), an MYC-type bHLH transcription factor, plays a vital role in activating CBFs expressions, which binds to MYC *cis*-element (CANNTG) in CBFs promoters under cold stress (Zarka et al. 2003). The expression of *CBF3* and many downstream cold-responsive genes are decreased in the *ice1* mutant lines in plant chilling and freezing tolerance, while up-regulated CBF regulon was found in the overexpressed ICE1 lines (Chinnusamy et al. 2003; Lee et al. 2005).

The mitogen-activated protein kinase (MAPK) cascades are fundamental signal molecules in eukaryotes, and the pathway involved in CBFs signal transduction. MAPK kinase-2 (MKK2) is activated by cold stress at 4 °C, and MPK4 and MPK6 are the direct downstream target of MKK2 (Teige et al. 2004). The constitutively active MKK2 in *Arabidopsis* lines induced CBF2/3 expression (Teige et al. 2004). Conversely, MPK3/6 phosphorylates and destabilizes ICE1 to inhibit the *CBF* expression and reduce freezing tolerance (Li et al. 2017). It also has been demonstrated that CBFs are negatively regulated by transcription factors such as ZAT12, MYB15 and PIF4/7 (Ding et al. 2019; Vogel et al. 2005). Taken together, the cold signal transductions and response in plants involved various messenger molecules and transcriptional, post-transcriptional and post-translational regulations, it is still needed to explore the molecular mechanisms of the fundamental cold signal sensing and response in the future.

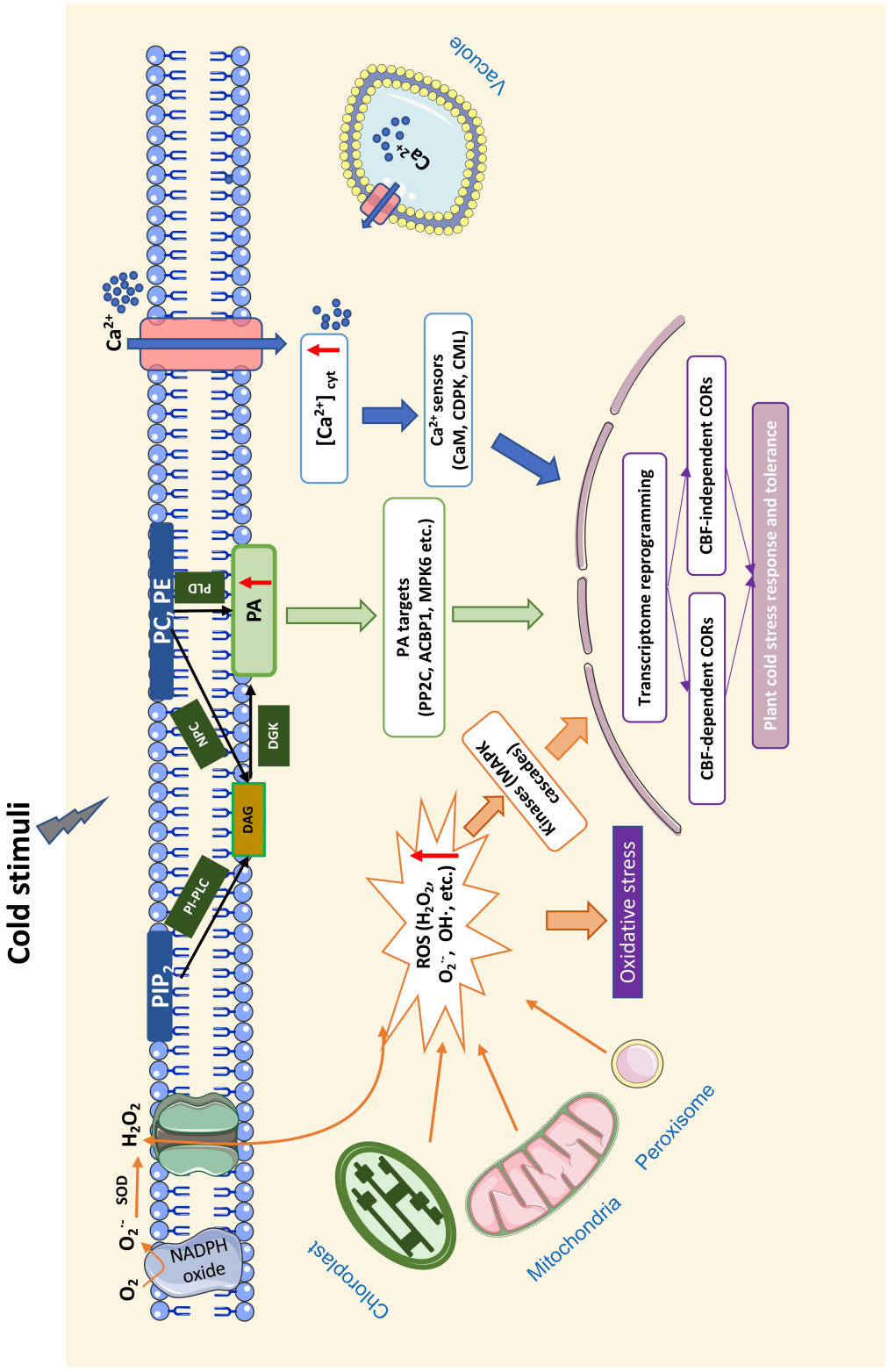
Cold stimuli
Vacuole
Ca2+
Ca2+
[Ca2+] cyt
Ca2+ sensors
(CaM, CDPK, CML)
PC, PE
PLD
PA
PA targets
(PP2C, ACBP1, MPK6 etc.)
NPC
DGK
DAG
PI-PLC
PIP2
Transcriptome reprogramming
CBF-independent CORs
CBF-dependent CORs
Plant cold stress response and tolerance
Kinases (MAPK cascades)
ROS (H2O2, O2·-, OH·, etc.)
Oxidative stress
H2O2
SOD
O2·-
O2
NADPH oxide
Chloroplast
Mitochondria
Peroxisome

Figure 1.2 Model of reactive oxygen species (ROS) generation, phosphatidic acid (PA) and calcium ion (Ca^{2+}) signaling pathways under cold stress in plant cells. Under cold stress, the chloroplast, mitochondria, peroxisome, and NADPH oxide contribute to generate ROS, which cause oxidative stress and cold stress response. The PA contents also increase dramatically by remodeling lipid compositions (such as PLD and PLC hydrolyzing pathways), PA could interact with various targets to alleviate cold stress. In addition, the increased cytosolic Ca^{2+} concentration will activate a variety of Ca^{2+} censors to regulate the downstream targets that ultimately enhance to the cold stress tolerance. ACBP: acyl-coenzyme A-binding proteins; $[Ca^{2+}]_{cyt}$: cytoplasmic Ca^{2+} concentration; CaM: calmodulin; CBF: C-repeat bind factors; CML: CaM-like proteins; CORs: cold-regulated genes; DAG: diacylglycerol; DGK: diacylglycerol kinase; MAPK/MPK: mitogen-activated protein kinase ; NPC: non-specific phospholipase C; PI: phosphatidylinositol; PI-PLC: phosphoinositide phospholipase C; PIP_2: phosphatidylinositol 4,5-bisphosphate; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PLC: phospholipase C; PLD: phospholipase D; PP2C: protein phosphatases 2C; SOD: superoxide dismutase.

1.2.4. Plant cold stress tolerance mechanisms

1.2.4.1.Protective proteins

Studies have demonstrated the accumulation of protective proteins including late embryogenesis abundant (LEA) proteins, ice-binding proteins (IBPs), heat shock proteins (HSPs) and cold shock domain (CSD) proteins during the cold acclimation process (Bredow et al. 2017; Karlson and Imai 2003; Timperio et al. 2008; Xu et al. 2020). It has been reported that LEA proteins are the major proteins and play a protective role in alleviating plant cold damage (Banerjee and Roychoudhury 2016). The COR proteins are a group of genes of the LEA-II group that were first observed by Guy et al. (1985) in spinach, which is involved in a wide range of low temperature responses, including cryoprotective protein, solutes biosynthesis, ROS detoxification, hormone metabolism, membrane transporter and signal transduction in growing plants (Chinnusamy et al. 2007; Fowler and Thomashow 2002; Maruyama et al. 2004). In addition, plants can synthesize IBPs to prevent ice nucleation, which was secreted from the cytoplasm into the apoplast and can absorb the ice and modify its growth at sub-zero temperatures (Bredow and Walker 2017), and they can prevent the recrystallization of extracellular ice (Davies 2014).

Cold shock proteins (CSPs) as RNA chaperones unwind RNA secondary structures and regulate transcription and translation in bacteria (Kim et al. 2009). It is found in eukaryote proteins as an RNA-binding domain called the cold shock domain (CSD). The CSD proteins play important roles in plant development and stress response. For instance, *AtCSP3* has been highly induced in a border region of roots under freezing stress in *Arabidopsis*, and reverse genetic analysis indicated that *AtCSP3* might enhance freezing tolerance mRNA translation during cold acclimation (Kim et al. 2009). The heat shock proteins (HSPs) act as molecular chaperones and function in preventing

protein aggregation by refolding denatured proteins (Timperio et al. 2008). Although HSPs are widely recognized for heat stress, accumulation of HSPs such as HSP70 and some small HSPs have also been found during low temperature stress (Khurana et al. 2013; Renaut et al. 2008; Ukaji et al. 1999).

1.2.4.2.Osmo-protectants

Cold stress causes osmotic stress in plants, which affects cellular processes, and some overlap with cold stress, including ROS generation and abscisic acid accumulation (Hassan et al. 2021; Upadhyaya et al. 2013). Osmo-protectants such as soluble sugars, polyamines, glycine betaine (GB) and proline (Singh et al. 2015) are considered ubiquitous and regulate the cellular water potential between solution and ice formed in the apoplast. At the same time, carbohydrate sugar, proline, polyamines and other compounds of the osmo-protectants also regulate different metabolic processes, such as protection of membranes, stabilizing proteins, and scavenging ROS in plant cells (Singh et al. 2015). Moreover, it is suggested that exogenous application of osmo-protectants or modification of its responsive genes are effective strategies to improve cold tolerance.

1.2.4.3.Antioxidants

The overaccumulation of ROS causes oxidative stress and plants require detoxifying systems comprising enzymatic and non-enzymatic antioxidants to balance the ROS production under cold stress (Kazemi-Shahandashti and Maali-Amiri 2018). Enzymatic antioxidants of plants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutamine peroxidase (GPX), monodehydroascorbate (MDHAR), dehydroascorbate reductase (DHAR), glutathione

reductase (GR) and alternative oxidase (AOX) (Apel and Hirt 2004). Glutamine (GSH), ascorbic acid (AsA), carotenoids and α-tocopherol belong to non-enzymatic antioxidants (Das and Roychoudhury 2014). Transcript levels of antioxidant enzymes are upregulated by cold stress conditions, and the transgenic approach for overexpression of antioxidant synthesis genes is considered an efficient approach to enhance cold tolerance for crops in the future (Das and Roychoudhury 2014).

1.2.5. Lipid remodeling and PA response under cold stress

Cellular membranes are dynamic structures that can help plants adapt to environmental changes, such as extreme temperatures by altering the lipid composition and fatty acid unsaturation levels (Barrero-Sicilia et al. 2017; Lamers et al. 2020). Moreover, plasma membranes play an essential role in signal recognition and transduction by hosting receptors and mediating protein–lipid interactions (de Jong and Munnik 2021). Cold stress can reduce the fluidity of cellular membranes thereby increasing rigidity, which is considered the first target for signaling transduction in plants (Ding et al. 2019).

Remodeling lipid composition is considered an effective strategy adopted by plants to maintain membrane stability and functions under cold stress (Barrero-Sicilia et al. 2017; Moellering et al. 2010; Nadeem et al. 2019b; Wu et al. 2020). Fatty acid desaturation of lipid membrane is positively correlated to plant cold stress tolerance (Chen and Thelen 2013). Fatty acid desaturates (FAD) introduce double bonds in the fatty acyl chains of lipids and are localized in the ER and plastids in plant membranes (Narayanan et al. 2020). The unsaturation of lipids under cold stress is also helpful in preventing freezing injury, such as expansion-induced lysis (Uemura and Steponkus 1989).

1.3. The role of phosphatidic acid in plant cold stress tolerance

Phosphatidic acid (PA) has been identified as a critical molecule in mediating cellular stress signal transduction in higher plants (Arisz et al. 2013; Yao and Xue 2018). Numerous studies have indicated an instantaneous increase in PA levels in growing plant membranes in response to drought (Frank et al. 2000), heat (Chen et al. 2010; Song et al. 2020), cold (Arisz et al. 2013), soil salinity (Munnik et al. 2000), soil acidity (Nadeem et al. 2019b), and pathogen attack (Adigun et al. 2021; Zhao et al. 2013). PA is the simplest type of glycerophospholipids, composed of phosphoryl glycerol esterified with two fatty acyl chains (*sn*-1 and *sn*-2) and phosphate at the third fatty acyl chain (*sn*-3) position (Pokotylo et al. 2018). The PA serves as a central precursor for glycerophospholipids, galactolipids, and triacylglycerol biosynthesis (Athenstaedt and Daum 1999; Carman and Henry 2007). As a signaling molecule, one key mode of PA action is interacting with protein targets in mediating catalytic activities and intracellular distribution in response to stresses (Hong et al. 2016).

Furthermore, as a signaling molecule, one key mode of PA action is interacting with protein targets in mediating catalytic activities and intracellular distribution in response to stresses (Hong et al. 2016). In recent years, numerous studies have demonstrated the role of individual isoforms of phospholipase in regulating PA formation under various stress conditions, and many PA-binding proteins were also identified *via* genetic and biochemistry approaches (Hou et al. 2016; Wang 2005; Zhang et al. 2004). There are two principal pathways for generating PA in plant during stress conditions and these include phospholipase D (PLD)-mediated hydrolysis of structural phospholipids and DAG kinase (DGK)-mediated phosphorylation of DAG cells (Figure 1.2 and 1.3) (Testerink and Munnik 2005; Wang 2005).

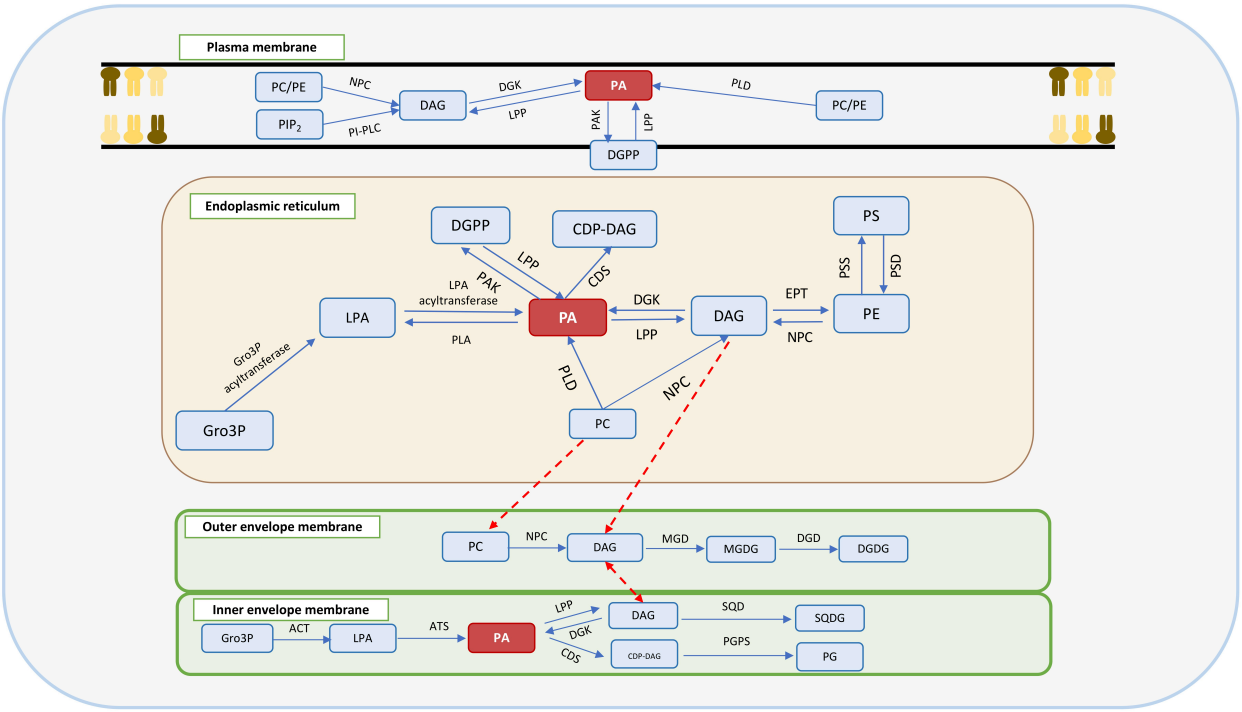

Figure 1.3 Model of phosphatidic acid biosynthesis pathways in the plant cell. There are several pathways for PA biosynthesis. The Gro3*P* is the main metabolic route, and the PLD and PLC-DGK pathways are activated to generate PA to acclimate cold stress.
Lipid transports are indicated by a red semi-dashed line. ACT: plastidial glycerpiol-3-phosphate acyltransferase; ATS: plastidial lysophosphatidic acid acyltransferase; CDP-DAG: cytidine diphosphate diacylglycerol; CDS: cytidine diphosphate diacylglycerol synthases; DAG: diacylglycerol; DGD: digalactosyldiacylglycerol synthetase; DGDG: digalactosyldiacylglycerol; DGK: diacylglycerol kinase; DGPP: diacylglycerol pyrophosphate; Gro3*P*: glycerpiol-3-phosphate; LPA: lysophosphatidic acid; MGD: monogalactosyl diacylglycerol synthase; MGDG: monogalactosyl diacylglycerol; NPC: non-specific phospholipase C; PA: phosphatidic acid; PAK: phosphatidic acid kinase; PAP: phosphatidic acid phosphatase; PC: phosphatidylcholine; PE: phosphatidylethanolamine; EPT: CDP-ethanolamine phosphotransferase; PG: phosphatidylglycerol; PGPS: phosphatidylglycerolphosphate phosphate synthase; PI: phosphatidylinositol; PI-PLC: phosphoinositide phospholipase C; PIP_2: phosphatidylinositol 4,5-bisphosphate; PLA: phospholipase A; PLC: phospholipase C; PLD: phospholipase D; PS: phosphatidylserine; PSD: PS decarboxylase; PSS: phosphatidylserine synthase; SQD: sulfoquinovosyl diacylglycerol synthase; SQDG: sulfoquinovosyl diacylglycerol.

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1.5. Structure of the thesis

This thesis follows a manuscript format and consist of four chapters:

Chapter 1: This chapter includes the rationale and overview; literature review, thesis objectives and hypothesis.

Chapter 2: The title of this chapter is "Effects of chilling stress on morphological, physiological and biochemical attributes of silage corn genotypes during seedling establishment". The variation in seedling growth, photosynthesis, oxidative stress levels, and antioxidant activities in two silage corn genotypes under different temperature regimes (25 °C, 20 °C, 15 °C, 10 °C and 5 °C) have been compared.

Chapter 3: The title of this chapter is "Remodeling of phopholipidome of silage corn by cold stress: a successful strategy to achieve cold tolerance involving phosphatidic acid". In this chapter, the lipidome profile of silage corn genotypes and investigated lipid remodeling in response to different temperature treatments at silage corn early growth stage. The lipid profiles of silage corn leaf and root in cold stress response using lipid chromatography-mass spectrometry based lipidomic platform have been analyzed. In addition, the transcriptional levels of different PA biosynthesis pathways between these two genotypes under low temperature regimes were studied.

Chapter 4: This chapter summarizes the overall results/discussion and conclusions. Further studies are suggested based on outcome of present studies for future direction.

Chapter 2

Effects of chilling stress on morphological, physiological and biochemical attributes of silage corn genotypes during seedling establishment

Abstract

Chilling stress is one of the major abiotic stresses which hinder seedling emergence and growth. Herein I investigated the effects of chilling/low temperature stress on the morphological, physiological, and biochemical attributes of two silage corn genotypes during the seedling establishment phase. The experiment was conducted in a growth chamber, and silage corn seedlings of Yukon-R and A4177G-RIB were grown at 25 °C up to V3 stage and then subjected to five temperature regimes (25 °C, 20 °C, 15 °C, 10 °C, and 5 °C) for 5 days. After the temperature treatment, the morphological, physiological, and biochemical parameters were recorded. Results indicated that temperatures of 15 °C and lower significantly affected seedling growth, photosynthesis system, reactive oxygen species (ROS) accumulation and antioxidant enzyme activities. Changes in seedlings growth parameters were in the order of 25 °C > 20 °C > 15 °C > 10 °C > 5 °C, irrespective of genotypes. The chlorophyll content, photosynthetic rate, and maximal photochemical efficiency of PS-II were drastically decreased under chilling conditions. Besides, chilling stress induced accumulation of hydrogen peroxide and malonaldehyde contents. Increased proline content and enzymatic antioxidants include superoxide dismutase, catalase and ascorbate peroxide were found to alleviate oxidative damage under chilling stress. However, the genotype of Yukon-R exhibited better adaption to chilling stress than A4177G3-RIB. Yukon-R showed significantly higher proline content, and enzymatic antioxidant activities than A4177G3-RIB under severe chilling conditions (temperature ≤ 10 °C). Similarly, Yukon-R expressed low temperature-induced ROS accumulation. Furthermore, the interaction effects were found between

temperature treatment and genotype on the ROS accumulation, proline content and antioxidant enzyme activities. In summary, the present study indicated that Yukon-R has shown better adaptation and resilience against chilling temperature stress, and therefore could be considered a potential candidate genotype to be grown in the boreal climate.

Keywords: Antioxidant, cold stress tolerance, plant growth, photosynthesis, root morphology, silage corn.

2.1. Introduction

Silage corn (*Zea mays* L.) is known as a high-energy crop, with higher biomass production potential, uniform growth quality, and one-cut harvest benefits (Ericsson and Nilsson 2006; Kirkland et al. 2005; Phipps et al. 2000). Besides, silage corn is highly palatable, digestible, and easy to ensile due to the high contents of soluble sugar (Khan et al. 2015). Due to these attributes, silage corn is mostly cultivated on large area as a forage crop (Cordeiro et al. 2019; Guyader et al. 2018). Corn originates from subtropical regions and is highly sensitive to chilling stress (0 – 20 °C) at all growth and development stages (Chinnusamy et al. 2007; Miedema 1982). However, chilling stress usually occurs during the short growing season in the boreal climate where the average seasonal growth temperature ranges from 10 to 12 °C, which is a major environmental factor limiting forage yield (Nadeem et al. 2019; Nadeem et al. 2020).

Chilling stress induces significant physiological and morphological changes in growing plants, including reduced seedling emergence, leaf initiation, disordered root cell division and elongation (Farooq et al. 2009; Mukhopadhyay and Roychoudhury 2018). The reduction in root architectural

growth also results in reduced water and nutrient uptake leading to reduced nutrient use efficiency (Grossnickle 2005). Moreover, chilling temperatures delay flowering, disturb the gametophyte and pollen development during reproductive growth, and such effects contribute to poor seed filling and seed development (Arshad et al. 2017; Thakur et al. 2010; Zinn et al. 2010).

Numerous studies indicated that plant photosynthetic capacity is inhibited by chilling stress, which can cause dysfunction of photosynthesis apparatus localized inside the chloroplast (Allen and Ort 2001; Long and Spence 2013). The stomatal closure has negative effects on the leaf gas exchange due to the limitation of CO_2 supply (Lianopoulou et al. 2014; Peeler and Naylor 1988). The low-temperature stress also decreases light absorption in the thylakoid electron transport of photosystem II (PS-II) and increases the excitation energy quenching in the light-harvesting antennae (Bilska and Sowiński 2010). Adam and Murthy (2014) and Sharma et al. (2020) demonstrated the decreased leaf maximum photochemical efficiency of PS-II (*Fv/Fm*), the efficiency of excitation energy transfer to open PS-II reaction center (*Fv'/Fm'*), and non-photochemical quenching coefficient (q_p) values and photoinhibition of PS-II in the growing plants under chilling stress.

Chilling stress also contributes to the over-accumulation of reactive oxygen species (ROS) in the growing plants (Zhang et al. 2021). Generally, ROS such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), and hydroxyl radical (OH•) are produced under chilling stress, and such ROS over-accumulation leads to plant oxidative stress (Castro et al. 2021; Chinnusamy et al. 2007; Gill and Tuteja 2010). It has been reported that lipid peroxidation increases membrane leakage, whereas it decreases membrane fluidity and is considered the main reason for damaging membrane-localized proteins impacting functions ofion channels, receptors, and enzymes (Das

and Roychoudhury 2014). Moreover, chilling stress affects the stability and solubility of proteins which leads to disrupted metabolic reactions for plant growth and development including the Calvin cycle (Siddiqui and Cavicchioli 2006).

Plants, therefore, attempt to mediate the adverse effects of temperature downshifts through stress tolerance defense mechanisms (Hassan et al. 2021). Plants increase their chilling stress tolerance by regulating cold-induced gene expressions, osmotic potential, membrane stabilization, and ROS scavenging activation (Miura and Furumoto 2013; Ritonga and Chen 2020). For instance, the C-repeat binding factor/dehydration-responsive element-binding protein 1 (CBF/DREB1) - dependent signal pathway was reported to show a complex regulation of the cold response network (Ding et al. 2019). For instance, the *CBF* genes are reported to bind to conversed CRT/DRE motifs (A/GCCGAC) in the promoter of *COR* genes and then induce their expression to enhance cold stress (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Stockinger et al. 1997). Biosynthesis of osmoprotectants such as soluble sugars, polyamines, glycine betaine (GB), and proline is also considered a strategy used by plants to adapt to chilling stress (Singh et al. 2015). In addition, plants require detoxifying systems comprising enzymatic and non-enzymatic antioxidants to reduce ROS accumulation during chilling stress conditions (Kazemi-Shahandashti and Maali-Amiri 2018). Enzymatic antioxidants of plants include superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxide (APX), glutathione peroxidase (GPX), and others (Das and Roychoudhury 2014). Previous studies showed that plant chilling tolerance capacity is linearly correlated with antioxidant systems, and the transcript levels of antioxidant enzymes are upregulated in chilling-tolerant varieties (Das and Roychoudhury 2014; Guo et al. 2006; Nejadsadeghi et al. 2014). These results indicate the crucial role of the antioxidant system in alleviating oxidative stress during chilling temperature conditions. In addition, low-temperature

stress tolerance varies within crop species and genotypes. Our previous studies showed that the agronomic performance varies in silage corn genotypes when evaluated at the physiological maturity under field conditions in the boreal climate (Ali et al. 2019a; Ali et al. 2019b; Nadeem et al. 2019). Early seedling establishment is an important step in crop growth and chilling stress is very common in boreal climate. There is limited literature available on plant growth, photosynthetic characteristics, and antioxidant activities in silage corn genotypes under low temperature/chilling stress during the early growing stages, particularly in boreal agro-ecosystem. Therefore, the aim of this study was to assess the effects of low temperature regimes on morphological, physiological and biochemical responses of two contrasting silage corn genotypes. Furthermore, we tested the relationships between the growth parameters and chilling stress tolerance parameters of silage corn genotypes. We hypothesized that chilling stress would alter root morphological traits, shoot growth, photosynthetic efficiency, redox homeostasis, and enzymatic antioxidants of silage corn. Moreover, different silage corn genotypes would express different of these traits under chilling stress at the early growth stage based on their chilling resistant levels.

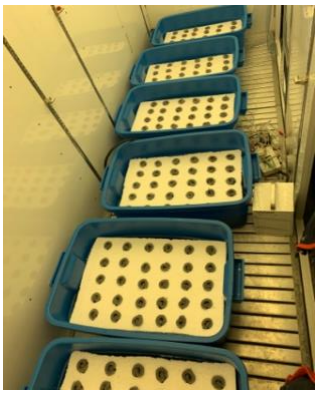
2.2.Materials and Methods

2.2.1. Experimental design, plant material, and growth conditions

The experiment was conducted in a walk-in growth chamber (BioChambers Inc., Winnipeg, MB, Canada) at Grenfell Campus, Memorial University of Newfoundland and Labrador, Canada in 2020/2021. The experiment was set up in a completely randomized design in a split-plot arrangement with three replications and repeated twice. The experimental treatments included two silage corn genotypes (Yukon-R and A4177G3-RIB) and five temperature levels (25 °C as control,

20 °C (non-chilling), and 15 °C, 10 °C and 5 °C were considered as chilling temperatures). Silage corn genotypes were selected based on our previous published work where we demonstrated that Yukon-R performed better than A4177G3-RIB in terms of agronomic performance (Ali et al, 2019b, Nadeem et al, 2019).

Equal sized silage corn seeds with homogeneous seed weight (0.24~0.28 g) were selected for the experiment. The sterilized silage corn seeds were then placed on a plastic tray with water-saturated tissue paper in the dark at 25 °C for germination (Huang et al. 2019). The seeds were allowed to germinate for 4 days, and water was sprayed daily to keep the tissue paper at a saturated moisture level. After the germination, the seedlings with uniform size were transplanted and anchored in a styrofoam board and transferred to three plastic containers (60 cm × 40 cm × 30 cm). These containers were used as deep water culture hydroponic systems containing half-strength Hoagland's nutrient solution (Hoagland and Arnon 1950). The pH and electric conductivity of the nutrient solution was maintained between 5.8−6.2 and 1.3-1.4 dS/m, respectively. Aerating the nutrient solution with air pumps, and nutrient solution was changed every fourth day. The growth chamber settings were tuned to 25 °C, with 14 h/10 h (light/dark) photoperiod, 65–70% relative humidity, and 300 μmol m^{-2} s^{-1} light intensity. The growth condition of silage corn seedlings was monitored daily, and temperature and humidity conditions of the growth chamber were recorded by the HOBO data logger (Onset Computer Corporation, MA, USA). At the V3 stage (10-day old seedling), the temperature regime was imposed according to the treatments (optimum or chilling stress). There were three containers for each temperature treatment, each container represents one replication with 20 seedlings (10 seedlings for each genotype).



Day 1

Day 5

Day 10 (V3 stage)

Figure 2.1 Experimental settings in growth chambers and silage corn seedling growth in plastic tubes in nutrient solution.

2.2.2. Plant growth and physiological performance

2.2.2.1.Chlorophyll contents

Leaf chlorophyll pigments (Chl *a* and Chl *b*) in growing seedling were recorded following the method developed by Minocha et al. (2009). Briefly, the fresh seedling leaves (100 mg) from each treatment were cut and submerged into 5 mL of 95% (v/v) ethanol in a dark place at 4 °C for 24 h. After that, the absorbance of extract solution was recorded spectrophotometrically with Cytation-5 microplate reader (BioTek Instruments, VT, USA) at 649 nm and 665 nm based on the equations used for Chl *a* and Chl *b* calculations. The chlorophyll contents were calculated according to the formulas of Lichtenthaler and Wellburn (1983) and expressed as μg g^{-1} fresh weight (FW):

$$Chl\ a\ (\mu g/mL) = 13.95 \times A665 - 6.88 \times A649$$

$$Chl\ b\ (\mu g/mL) = 24.96 \times A649 - 7.32 \times A665$$

2.2.2.2.Plant growth and root characteristics

For seedling growth parameters, seedlings were harvested and divided into shoots and roots. Seedling length, seedling fresh weight, and root fresh weights were measured and recorded. Thereafter, the roots were scanned with an Epson Perfection V850 Pro root scanner (Regent Instruments Inc., QC, Canada) at a resolution of 600 dpi. Root morphological traits including root length, surface area, root volume, average root diameter, root tips, root crossings, and root forks were measured by the WinRHIZO™ Pro image analysis system (Regent Instruments Inc., QC, Canada). The shoots and roots were then subsequently dried in a forced air oven (Sheldon Manufacturing Inc. Cornelius, OR, USA) at 65 °C for 72 h and dry weight was measured.

2.2.2.3.Photosynthesis rate and maximum photochemical efficiency of photosystem-II

Photosynthesis rate was measured from the second fully expanded seedling leaf from the top using a portable photosynthesis system (LI-COR 6400XT, LI-COR BioSciences, NE, USA) maintaining the leaf chamber temperature, air relative humidity, CO_2 concentration, and photosynthetic photon flux density (PPFD) at 20 °C, 80–90%, 400 μmol mol^{-1}, and 1000 μmol m^{-2} s^{-1}, respectively. The maximum photosystem II quantum yield (F_v / F_m = (F_m - F_0) / F_m) of the seedling leaves was recorded using LI-6400XT with leaf chamber fluorometer after 30 min of dark adaption (Salesse-Smith et al. 2020).

2.2.3. Biochemical analyses

2.2.3.1. Proline contents

Seedling leaf tissues (500 mg) were mixed with 5 mL of 3% (w/v) sulfosalicylic acid in 50 mL test tubes incubated at 100 °C in a water bath for 10 min (Wehner et al. 2016). A 2 mL aliquot of the cooled mixture was added to a mixture of 2 mL acetic acid and 3 mL ninhydrin solution, and the mixture was heated at 100 °C for 40 min. After cooling, 4 mL toluene was added to each tube. The tube was then closed with a stopper and vortexed for 30 s followed by 60 min incubation in the dark. The proline–toluene phase (upper layer) was collected into a fresh tube, and the absorbance was measured at 520 nm using a Cytation-5 microplate reader. Proline contents were expressed as μg g^{-1} FW and calculated according to the proline standard curve (concentration between 0-20 μg/mL):

$$y = 0.014 \times x - 0.029 \quad R^2 = 0.997$$

2.2.3.2. Hydrogen peroxide and malondialdehyde contents

Hydrogen peroxide (H_2O_2) content of seedling leaf was determined as described by Othman et al. (2017) with slight modifications. Briefly, seedling leaf samples (200 mg) were ground in 1.5 mL 0.1% trichloroacetic acid (TCA) solution, and the homogenate was centrifuged at 15,000 g for 20 min at 4 °C. Next, 0.5 mL supernatant was mixed with 0.5 mL 10 mM phosphate-buffered saline (PBS) solution (pH 7.0) and 1 mL 1M potassium iodide (KI) solution in a 2 mL centrifuge tube and then incubated at 28 °C. After 60 min of incubation, the absorbance at 390 nm wavelength was recorded in a microplate reader, and the H_2O_2 contents (μmol H_2O_2 g^{-1} FW) were calculated according to the following standard curve (concentration between 0-100 nmol/mL):

$$y = 5.54 \times x - 0.004 \quad R^2 = 0.999$$

Lipid peroxidation was determined by quantifying the malondialdehyde (MDA) content in leaf samples by adopting the method of Hodges et al. (1999). Briefly, 500 mg seedling leaf samples were homogenized in 5 mL 10% (w/v) TCA and then centrifuged at 12,000 g for 20 min at 4 °C. Then 2 mL supernatant was added into 2 mL 0.67% (w/v) thiobarbituric acid (TBA), then the mixture was kept in the boiling water for 15 min. The mixture was quickly chilled in the ice bath to stop the reaction. Afterward, the mixture was centrifuged at 15,000 g for 15 min. The absorbance was recorded at 440, 532, and 600 nm by a Cytation-5 microplate reader, and the MDA concentration (μmol L^{-1}) was calculated adopting the following formula:

$$cMDA = 6.452 \times (A532 - A600) - 0.56 \times A440$$

2.2.3.3. Protein extraction and antioxidant enzyme assays

For crude protein extraction, 200 mg seedling leaf sample was homogenized in 1.5 mL extraction buffer, which contained 0.1 M PBS (pH 7.8), 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ascorbic acid and 1% (w/v) polyvinylpyrrolidone (PVP) using prechilled mortar and pestle. The homogenate was centrifuged at 12,000 RPM for 20 min at 4 °C, and the supernatant was collected for the protein assay and enzyme activity measurements as explained below.

The protein content in seedling leaf samples was analyzed by a Cytation-5 microplate reader following the protocol of Bradford (1976). Briefly, 0.1 mL of crude protein supernatant was pipetted into a test tube, 5 mL of Coomassie Brilliant Blue G-250 protein reagent was added. The contents were mixed well by shaking and then standing for 2-3 min. Protein concentration (mg g^{-1} FW) was determined at the 595 nm wavelength with the standard curve (concentration between 0-100 μg/mL):

$$y = 0.000528 \times x - 0.00456 \quad R^2 = 0.994$$

The method developed by Beauchamp and Fridovich (1971) was followed to determine superoxide dismutase (SOD, EC. 1.15.1.1) activity in corn leaves. One unit of SOD activity was defined as causing 50% inhibition of nitro blue tetrazolium (NBT) at 560 nm wavelength. The 40 μL enzyme extract was added into a 3 mL reaction mixture (50 mM PBS (pH 7.8), 2.25 mM NBT, 60 μM riboflavin, 3 mM EDTA, 14.5 mM methionine) and the reaction was conducted in a light incubator under 4000 lux at 25 °C for 20 min. The unit of SOD activity was expressed in unit mg^{-1} protein.

The assay of catalase (CAT, EC. 1.11.1.6) activity was conducted following the method developed by Cakmak and Marschner (1992). Briefly, 0.1 mL of enzyme extract was added to the 2.9 mL

reaction mixture containing 0.15 M PBS (pH 7.0) and 10 mM H_2O_2. The absorbance at 240 nm was recorded for 120 s in a UV-visible spectrophotometer (Agilent Technologies Inc, CA, USA). The CAT activity was expressed in the decomposition of 1 μmol of H_2O_2 per minute (μmol mg^{-1} protein min^{-1}).

Ascorbate peroxidase (APX, EC. 1.11.1.11) activity was measured according to the method developed by Nakano and Asada (1981). The reaction mixture was prepared by adding PBS (50 mM, pH 7.0), EDTA (0.1 mM), ascorbic acid (AsA, 5 mM), and H_2O_2 (20 mM), and then, 0.1 mL of supernatant was added into a 2.9 mL reaction mixture. The absorbance at 290 nm was recorded for 120 s in a UV-visible spectrophotometer (Agilent Technologies Inc, CA, USA). The APX activity was expressed in the amount of enzyme that can oxidase 1 μmol of AsA per minute (μmol mg^{-1} protein min^{-1}).

2.2.4. Statistical analyses

The experimental design was as follows: There were three biological replicates for each experimental unit and each biological replicate had at least three technical replicates. The experiment was repeated twice independently. Both independent experiments expressed the same trends, therefore, the data were pooled and analyzed. The Shapiro-Wilk test was employed on the data to check the normality before further statistical analysis was conducted. The XLSTAT program (Permium 2020, Addinsoft Inc, NY, USA) was used for two-way analysis of variance (ANOVA) with three sources of variation (genotypes, temperatures, and their interactions) and means were compared using Fisher's Least Significant test ($p \leq 0.05$). All data in this study were expressed as means ± standard errors (SEs). Figures were prepared using GraphPad Prism (version 8.4.3, GraphPad Software, CA, USA). Principal Component Analysis (PCA) and its visualization

were performed using the XLSTAT program to determine the relationships between temperatures, genotypes, morphological, physiological, and biochemical attributes. R statistic software was used to conduct Pearson's correlation analysis (R Core Team 2021).

2.3. Results

2.3.1. Seedling growth and root characteristics

Temperature regime significantly (p<0.001) affected the seedling shoot length, shoot dry weight, root dry weight, and seedling dry weight of silage corn genotypes. However, the interaction was only significant in the case of shoot length (Table 2.1). Significantly higher shoot dry weight (0.95±0.02 g seedling^{-1}), root dry weight (0.25±0.01 g seedling^{-1}), and seedling dry weights (1.20±0.03 g seedling^{-1}) were recorded at 25 °C, whereas the lowest values were observed at 5 °C (Table 2.1). Overall, the evaluated growth parameters varied in order of 25 °C > 20 °C > 15 °C > 10 °C > 5 °C (Table 2.1). Yukon-R silage corn genotype produced seedlings with significantly higher shoot dry weight (0.75±0.03 g seedling^{-1}), root dry weight (0.22±0.01 g seedling^{-1}), and total dry weight (0.97±0.03 g seedling^{-1}) compared to genotype A4177G3-RIB. (Table 2.1). Interaction among growth temperature and silage corn genotypes displayed maximum shoot length (80.02±1.30 cm seedling^{-1}) in Yukon-R when grown at 25 °C, while the lowest shoot length (44.99±0.52 cm seedling^{-1}) was noted in A4177G3-RIB at 5 °C (Table 2.1).

Temperature regimes significantly (p<0.05) affected the total root length, individual root length (0–0.5 mm and >0.5 mm), total root volume and individual root volume (0 – 0.5 mm and > 0.5 mm), total root surface area and individual root surface area (0 – 0.5 mm and > 0.5 mm), total root forks and total root crossings of silage corn genotypes (Table 2.22.3). The interactions between growth temperature and silage genotype were significant in root length (> 0.5 mm), root volume

(0 – 0.5 mm, > 0.5 mm), and root surface area (0 – 0.5 mm and > 0.5 mm) as shown in Table 2. Silage corn seedlings grown at 25 °C had significantly longer roots (1416±59 cm seedling^{-1}), higher root volume (2.24±0.05 cm^3 seedling^{-1}), total root surface area (158.19±3.19 cm^2 seedling^{-1}), average root diameter (0.366±0.007 mm seedling^{-1}), total root tips (3649±358 seedling^{-1}), root tips at 0 – 0.5 mm (3592±346 seedling^{-1}), total root forks (5341±211 seedling^{-1}) and total root crossings (1298±65 seedling^{-1}) whereas the lowest values were noticed at 5 °C, irrespective of silage corn genotypes (Table 2.2-2.3, Figure 2.2). Yukon-R had significantly longer roots (1108±69 cm seedling^{-1}), higher root volume (1.48±0.06 cm^3 seedling^{-1}), total root surface area (104.55±6.19 cm^2 seedling^{-1}), higher root forks (4090±290 seedling^{-1}) and root crossings (1009±79 seedling^{-1}) than A4177G3-RIB (Table 2.2-2.3). Overall, all the root traits varied in order of the following temperature: 25 °C > 20 °C > 15 °C > 10 °C and > 5 °C, irrespective of silage corn genotypes. However, Yukon-R showed superior shoot growth and root morphological traits than A4177G3-RIB (Table 2.2-2.3, Figure 2.2).

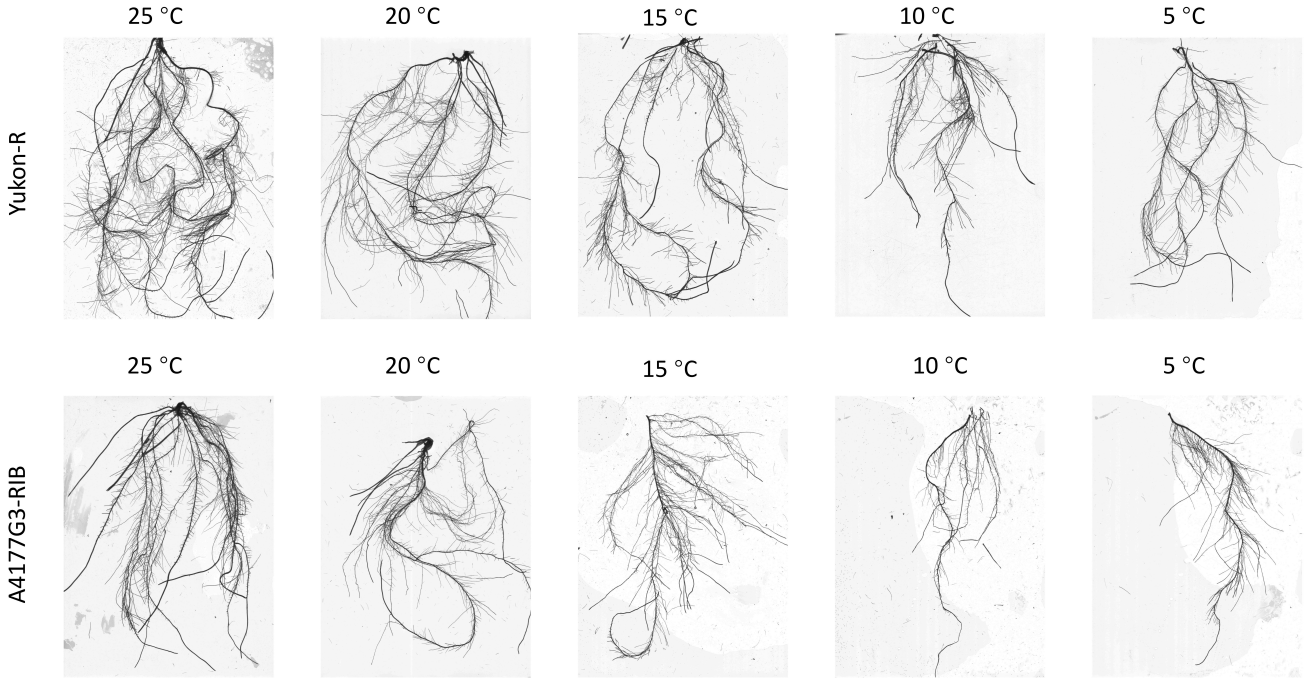


Figure 2.2 Effect of chilling temperature (5, 10 and 15 °C), and non-chilling temperature (20 and 25 °C) on silage corn seedling root growth of Yukon-R and A4177G3-RIB.

Table 2.1 Effects of different temperature regime on seedling shoot length, shoot dry weight, root dry weight, and total seedling dry weight of two silage corn genotypes cultivated in hydroponics under controlled environment.

Source of variance	Seedling shoot length (cm^{-1})	Shoot dry weight (g^{-1})	Root dry weight (g^{-1})	Seedling dry weight (g^{-1})
Temperature (Temp)				
25 °C (control)	**77.41±1.14 a**	**0.95±0.02 a**	**0.25±0.01 a**	**1.20±0.03 a**
20 °C	72.66±1.00 b	0.79±0.01 b	0.22±0.01 b	1.02±0.02 b
15 °C	63.93±1.84 c	0.70±0.02 c	0.19±0.01 c	0.89±0.03 c
10 °C	53.79±1.04 d	0.60±0.02 d	0.16±0.01 d	0.75±0.03 d
5 °C	47.42±0.95 e	0.49±0.02 e	0.15±0.01 e	0.64±0.03 e
Genotypes (Gen)				
Yukon-R	**65.71±2.11 a**	**0.75±0.03 a**	**0.22±0.01 a**	**0.97±0.03 a**
A4177G3-RIB	60.37±2.21 b	0.66±0.03 b	0.16±0.01 b	0.83±0.04 b
Temp × Gen				
25 °C × Yukon-R	**80.02±1.30 a**	0.98±0.03	0.27±0.01	1.25±0.02
20 °C ×Yukon-R	73.25±1.14 b	0.82±0.03	0.25±0.01	1.07±0.03
15 °C × Yukon-R	68.74±1.73 c	0.74±0.02	0.23±0.01	0.97±0.02
10 °C × Yukon-R	56.71±1.12 d	0.65±0.02	0.19±0.01	0.83±0.02
5 °C × Yukon-R	49.87±1.34 e	0.56±0.02	0.18±0.01	0.74±0.02
25 °C × A4177G3-RIB	74.80±1.96 b	0.91±0.03	0.23±0.01	1.14±0.03
20 °C × A4177G3-RIB	72.07±0.99 bc	0.78±0.01	0.19±0.01	0.96±0.01
15 °C × A4177G3-RIB	59.12±0.58 d	0.67±0.02	0.16±0.003	0.82±0.02
10 °C × A4177G3-RIB	50.87±1.17 e	0.54±0.01	0.13±0.004	0.68±0.01
5 °C × A4177G3-RIB	44.99±0.52 f	0.43±0.01	0.12±0.003	0.54±0.01
Significance				
Temperatures	***	***	***	***
Genotypes	***	**	***	***
Temp × Gen	*	NS	NS	NS

*, **, *** represent significant differences at alpha 0.05, 0.01 and 0.001, respectively. The values present here are means ± standard errors (n=30 for Geno, n=12 for Temp, n=6 for Geno × Temp). Different letters within each column indicate significant differences among temperature regime, silage corn genotypes, or their interaction according to Fishers' Least Significant test (two-way ANOVA, p=0.05)

Table 2.2 Analysis of variance and mean comparisons for total root length, total root volume, and total surface area of silage corn genotypes, grown in different temperatures and their interaction under controlled environment.

Source of variance	Total root length (RL) (cm seedling $^{-1}$)	Root length 0-0.5 mm (cm seedling $^{-1}$)	Root length >0.5 mm (cm seedling $^{-1}$)	Total Root volume (RV) (cm^3 seedling $^{-1}$)	Root volume 0-0.5 mm (cm^3 seedling $^{-1}$)	Root volume >0.5 mm (cm^3 seedling $^{-1}$)	Total surface area (SA) (cm^2 seedling $^{-1}$)	Surface area 0-0.5 mm (cm^2 seedling $^{-1}$)	Surface area >0.5 mm (cm^2 seedling $^{-1}$)
Temperature (Temp)									
25 °C (control)	**1416±59 a**	**1145±59 a**	**271±8 a**	**2.24±0.05 a**	**0.42±0.02 a**	**1.82±0.03 a**	**158.19±3.88 a**	**64.21±3.19 a**	**77.23±2.54 a**
20 °C	1291±49 a	1082±47 a	209±7 a	1.50±0.07 b	0.36±0.02 b	1.14±0.08 b	121.31±6.82 b	59.89±3.81 a	47.79±3.49 b
15 °C	966±58 b	775±50 b	189±8 c	1.30±0.05 b	0.33±0.03 b	0.97±0.05 c	109.33±5.64 c	51.07±3.88 b	46.28±2.05 b
10 °C	612±48 c	484±45 c	127±5 d	0.80±0.03 c	0.19±0.01 c	0.61±0.03 d	66.68±3.06 d	29.19±2.09 c	30.13±1.38 c
5 °C	589±48 c	472±35 c	116±13 d	0.75±0.04 c	0.17±0.01 c	0.58±0.06 d	61.45±4.52 d	27.25±1.68 c	27.85±3.11 c
Genotypes (Gen)									
Yukon-R	**1108±69 a**	**906±60 a**	**201±9 a**	**1.48±0.06 a**	**0.34±0.02 a**	**1.14±0.08 a**	**104.55±6.19 a**	**54.22±3.56 a**	**50.33±2.95 a**
A4177G3-RIB	842±64 b	677±52 b	164±12 b	1.15±0.06 b	0.24±0.02 b	0.91±0.09 b	79.80±6.41 b	38.42±2.61 b	41.38±4.08 b
Temp × Gen									
25 °C × Yukon-R	1594±41	1302±41	**272±15 a**	2.33±0.08	**0.47±0.02 a**	**1.86±0.03 a**	148.04±3.54	**72.56±3.65 a**	75.47±2.43 a
20 °C ×Yukon-R	1513±59	1182±67	223±9 b	1.73±0.08	0.43±0.02 ab	1.31±0.11 b	125.04±5.56	70.52±2.99 a	54.52±3.66 b
15 °C × Yukon-R	1047±66	904±54	213±7 bc	1.45±0.06	0.41±0.02 b	1.09±0.05 c	113.62±4.95	62.07±3.03 b	51.56±2.09 b
10 °C × Yukon-R	848±33	581±68	140±9 e	0.88±0.08	0.22±0.02 ef	0.66±0.02 fg	67.33±3.19	34.56±2.56 de	32.77±1.26 de
5 °C × Yukon-R	608±60	562±47	158±8 de	0.95±0.04	0.19±0.01 fg	0.77±0.03 ef	68.73±3.54	31.38±2.24 e	37.35±1.50 cd
25 °C × A4177G3-RIB	1284±42	988±65	269±6 a	2.41±0.03	0.36±0.02 c	1.79±0.06 a	134.85±6.02	55.86±1.89 bc	**78.99±4.60 a**
20 °C × A4177G3-RIB	1186±69	981±38	195±6 c	1.27±0.04	0.30±0.02 d	0.98±0.09 cd	90.306±7.24	49.25±3.11 c	41.05±4.71 c
15 °C × A4177G3-RIB	765±25	647±42	166±7 d	1.11±0.03	0.26±0.02 de	0.85±0.04 de	81.09±3.16	40.07±2.95 d	41.01±1.72 c
10 °C × A4177G3-RIB	476±54	387±22	115±6 f	0.71±0.02	0.15±0.01 g	0.55±0.04 g	51.29±2.31	23.81±1.06 f	27.48±2.00 e
5 °C × A4177G3-RIB	429±18	343±14	74±7 g	0.54±0.02	0.14±0.0 g	0.39±0.05 h	41.47±1.81	23.11±0.79 f	18.36±2.09 f
Significance									
Temperatures	***	***	***	***	***	***	***	***	***
Genotypes	***	***	***	***	***	***	***	***	***
Temp × Gen	NS	NS	***	NS	***	*	NS	*	***

*, *** represents significant differences at alpha 0.05 and 0.001. The values present here are means ± standard errors (n=30 for Geno, n=12 for Temp, n=6 for Geno × Temp). Different letters within each column indicate significant differences among five growth temperatures, two silage corn genotypes, or their interaction according to Fishers' Least Significant test (two-way ANOVA, p=0.05).

Table 2.3 Analysis of variance and mean comparisons for root average diameters, total root tips, root forks, and root crossings of silage corn genotypes grown at different temperatures under a controlled environment.

Source of variance	Root average diameters (mm)	Total root tips	Root tips 0-0.05 mm	Root tips >0.05 mm	Root forks	Root crossings
Temperature (Temp)						
25 °C (control)	**0.366±0.007 a**	**3649±358 a**	**3592±346 a**	**39±4 a**	**5341±211 a**	**1298±65 a**
20 °C	0.352±0.004 a	2705±144 b	2685±144 b	19±2 b	4702±290 b	1233±69 a
15 °C	0.350±0.005 ab	2569±222 b	2566±220 b	30±2 c	3908±242 c	922±65 b
10 °C	0.350±0.009 ab	1794±168 c	1778±167 c	16±3 c	2106±166 d	427±41 c
5 °C	0.332±0.006 b	1684±127 c	1676±126 c	18±2 c	1933±124 d	411±43 c
Genotypes (Gen)						
Yukon-R	0.35±0.004	2665±227	2631±222	27±2	**4090±290 a**	**1009±79 a**
A4177G3-RIB	0.34±0.004	2310±145	2288±143	22±2	3107±258 b	707±69 b
Temp × Gen						
25 °C × Yukon-R	0.376±0.004	4248±495	4174±472	37±6	5711±238	1461±58
20 °C ×Yukon-R	0.357±0.004	2677±276	2611±274	16±2	5351±331	1391±86
15 °C × Yukon-R	0.347±0.005	2915±349	2881±345	34±4	4632±138	1119±38
10 °C × Yukon-R	0.342±0.016	1652±267	1630±264	21±5	2508±211	539±41
5 °C × Yukon-R	0.343±0.009	1833±175	1809±173	23±2	2245±167	534±48
25 °C × A4177G3-RIB	0.356±0.014	3050±419	3009±412	40±7	4971±291	1134±68
20 °C × A4177G3-RIB	0.343±0.005	2732±125	2710±125	21±3	4054±305	1075±60
15 °C × A4177G3-RIB	0.356±0.008	2277±234	2251±233	26±2	3184±174	724±41
10 °C × A4177G3-RIB	0.357±0.008	1937±212	1926±211	10±1	1703±112	314±27
5 °C × A4177G3-RIB	0.321±0.005	1556±181	1542±180	13±2	1621±33	288±4
Significance						
Temperatures	*	***	***	***	***	***
Genotypes	NS	NS	NS	NS	***	***
Temp × Gen	NS	NS	NS	NS	NS	NS

*, *** represents significant differences at alpha 0.001. The values present here are means ± standard errors (n=30 for Geno, n=12 for Temp, n=6 for Geno × Temp). Different letters within each column indicate significant differences among five growth temperatures, two silage corn genotypes, or their interaction according to Fishers' Least Significant test (two-way ANOVA, p=0.05).

2.3.2. Chlorophyll content, photosynthesis rate, and maximum photochemical efficiency of PS-II

Growth temperatures had significant effects ($p<0.001$) on total chlorophyll content, and Chl *b* content on silage corn seedlings, whereas Chl *a* content is affected ($p<0.001$) by growth temperatures only (Figure 2.3A-E). However, the interactions between growth temperatures and silage corn genotypes had no effects on Chl *a*, Chl *b,* and total chlorophyll contents (data not shown). Significantly higher total chlorophyll (1687.62±32.83 μg g^{-1} FW), Chl *a* (996.58±23.95 μg g^{-1} FW), and Chl *b* (696.32±11.38 μg g^{-1} FW) were observed at 25 °C whereas the lowest values were recorded at 5 °C (Figure 2.3A-C). Yukon-R genotype had significantly higher total chlorophyll (1241.73±57.38 μg g^{-1} FW) and Chl *b* (444.67±26.33 μg g^{-1} FW) than genotype A1477G3-RIB (Figure 2.3 D&E).

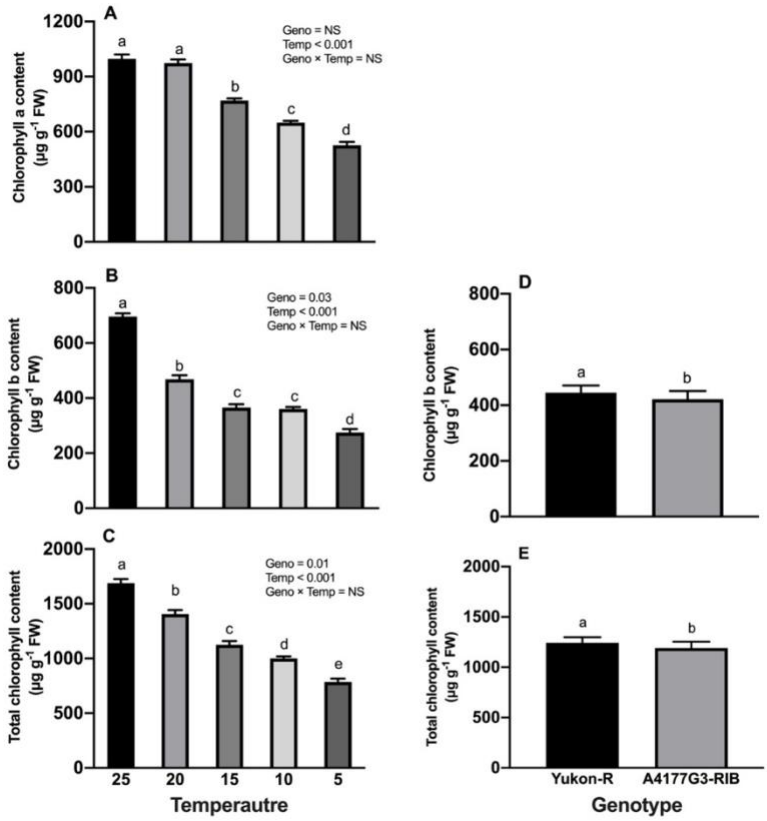


Figure 2.3 Effects of temperature regime on leaf chlorophyll (**A**), chlorophyll b (**B**), and total chlorophyll content (**C**) of silage corn genotypes, chlorophyll b content (**D**), and total chlorophyll content (**E**) observed in Yukon-R and A4177G3-RIB. Each vertical bar represents the average of replicates ± SE (n=30 for Geno, n=12 for Temp, n=6 for Geno × Temp). Different letters indicate significant differences among treatments at $p \leq 0.05$ according to Fisher's Least Significant test. Geno = genotype; Temp = temperature.

In this study, photosynthesis rate is a measure of net CO_2 assimilation rate of silage corn leaf, which is computed as the rate of CO_2 uptake per unit time per unit leaf area (μmol m^{-2} s^{-1}). Growth temperatures expressed significant (p<0.001) effects on the photosynthesis rate of silage corn genotypes (Figure 2.4B); however, the interaction (temperature regime × corn genotypes) had significant effects on the photosynthetic efficiency of PS-II (Figure 2.4C). A significantly higher photosynthesis rate (18.60±0.23 μmol m^{-2} s^{-1}) was observed at 25 °C, whereas the lowest was recorded at 5 °C (6.68±0.30 μmol m^{-2} s^{-1}), irrespective of silage corn genotypes (Figure 2.4A). Yukon-R showed a significantly higher photosynthesis rate (13.51±0.77 μmol m^{-2} s^{-1}) than A4177G3-RIB (12.14±0.85 μmol m^{-2} s^{-1}) (Figure 2.4B). Temperature × corn genotype interaction resulted in significantly higher *Fv/Fm* values in Yukon-R (0.75±0.01) at 25 °C. However, both genotypes had similar *Fv/Fm* values when exposed to 20 °C (Figure 2.4C).

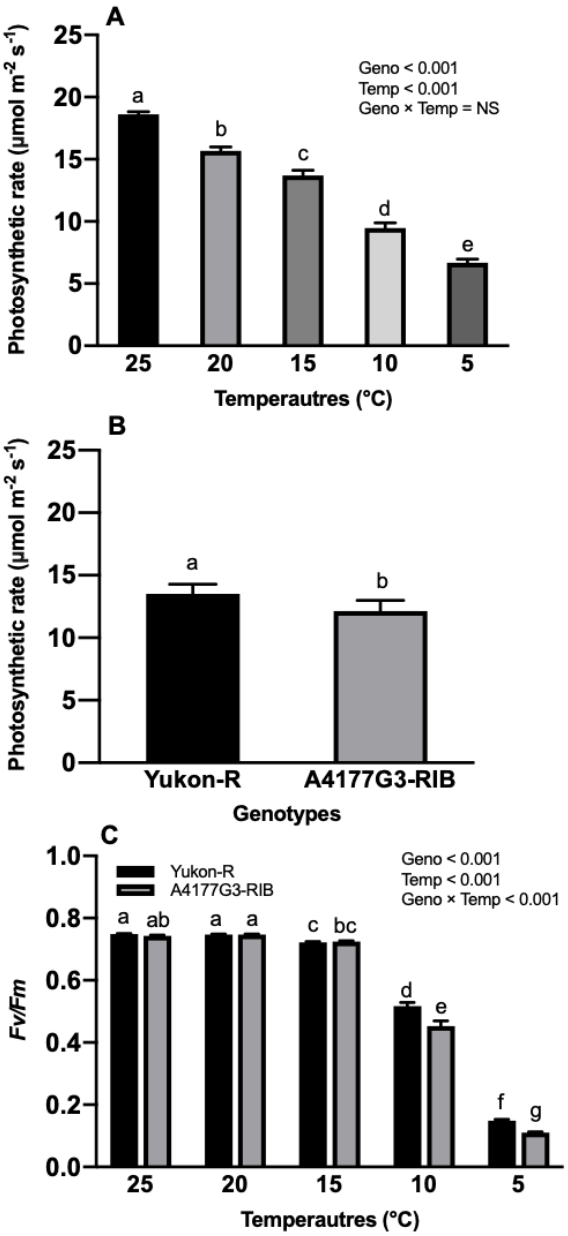


Figure 2.4 Effects of temperature regime on photosynthetic rate of silage corn (**A**), comparative photosynthesis rate of silage corn genotypes (**B**). Interactive effects of temperature and silage corn genotypes on photochemical efficiency of PSII (*Fv/Fm*) (**C**). Each vertical bar represents the average of replicates ± SE (n=30 for Geno, n=12 for Temp, n=6 for Geno × Temp). Different lowercase letters indicate significant differences among treatments at $p \leq 0.05$ according to Fisher's Least Significant test. Geno = genotype; Temp = temperature.

2.3.3. Hydrogen peroxide and malondialdehyde contents

The growth temperature, silage corn genotype, and their interactions (Geno × Temp) showed significant difference (p<0.001) in both H_2O_2 and MDA contents of silage corn seedlings. Significantly higher (831.80±11.91 nmol g^{-1} FW) H_2O_2 accumulation was also observed in A4177G3-RIB seedlings at 5 °C whereas the lowest (199.39±5.93 nmol g^{-1} FW) was noticed in the same genotypes at 25 °C, as shown in Figure 2.5A. Similarly, the MDA content was significantly higher (24.23±0.18 nmol g^{-1} FW) in A4177G3-RIB at 5 °C and the lowest (4.47±0.18 nmol g^{-1} FW) was recorded in the same genotype at 25 °C (Figure 2.5B). Both genotypes produced statistically similar H_2O_2 and MDA contents at 25 and 20 °C conditions, however, the MDA contents increased dramatically at 15°C. While comparing the main effects, genotypes expressed higher H_2O_2 and MDA contents in order of 25 °C > 20 °C > 15 °C > 10 °C and > 5 °C whereas A4177G3-RIB expressed higher content than Yukon-R (data not shown).

2.3.4. Proline contents and antioxidant enzyme activities

Temperature regime, silage corn genotypes, and their interaction (Geno × Temp) had significant (p<0.001) effects on proline contents (Figure 2.5C). Significantly higher proline content (148.58±11.18 ug g^{-1} FW) was observed in Yukon-R when grown at 5 °C, whereas the lowest was recorded in A4177G3-RIB at 20 °C (51.49±2.48 ug g^{-1} FW) (Figure 2.5C). Proline contents were observed in the order of 5 °C < 10°C < 15 °C < 20 °C and 25 °C while comparing the effects of temperature regime, and Yukon-R expressed higher proline content compared to A4177G3-RIB (data not shown).

Temperature regimes, silage corn genotypes, and their interaction (Geno × Temp) expressed significant (p<0.001) effects on antioxidants (CAT, APX, and SOD) (Figure 4D-F). Interactive

effects of temperature regime and silage corn genotypes displayed significantly higher CAT (16.23±0.76 μmol mg^{-1} protein min^{-1}), APX (22.89±1.61 μmol mg^{-1} protein min^{-1}), and SOD (6.18 ± 0.25 unit mg^{-1} protein) values in Yukon-R genotype when grown at 5 °C (Figure 2.5D-F). The lowest CAT activity (3.0±0.14 μmol mg^{-1} protein min^{-1}) was found at 20 °C in A4177G3-RIB, whereas the Yukon-R had the lowest SOD (2.60±0.07 unit mg^{-1} protein), APX (3.06±0.42 μmol mg^{-1} protein min^{-1}) activities at 25 °C. While comparing the main effects, CAT, APX and SOD varied in order of 5 °C > 10 °C > 15 °C > 20 °C > 25 °C, irrespective of silage corn genotypes (Figure 2.5D-F).

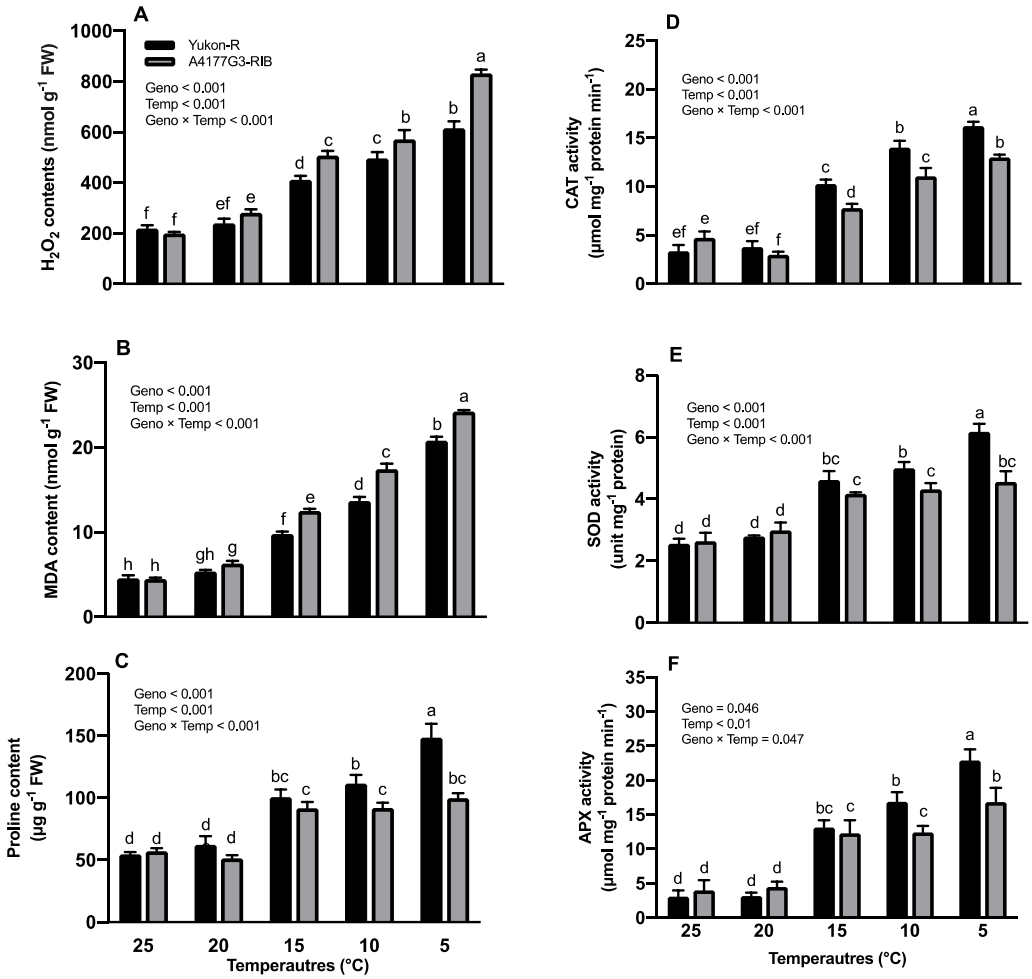


Figure 2.5 Interactive effects of temperature regime and silage corn genotypes on leaf H_2O_2 content (**A**), MDA contents (**B**), proline content (**C**), SOD activity (**D**), CAT activity (**E**), and APX activity (**F**). Each vertical bar represents the average of replicates ± SE (n=6). Different lowercase letters indicate significant differences among treatments at $p \leq 0.05$ according to Fisher's Least Significant test. Geno = genotype; Temp = temperature. H_2O_2, MDA, SOD, CAT, and APX represent hydrogen peroxide, malondialdehyde, superoxide dismutase, catalyze activity, and ascorbate peroxidase.

2.3.5. Relationships between morphological, physiological, and biochemical attributes of silage corn genotypes as influenced by different temperature regimes

A PCA was performed to assess the association between experimental treatments and morphological, physiological, and biochemical attributes of corn genotypes. The PCA explained 84.85% variability in the total data, where the first dimension (F1) displayed 78.38% and the second dimension (F2) showed 6.48% variability, respectively (Figure 2.6A&B). The studied parameters grouped temperature regimes in different quadrants (Figure 2.6A&B). The 21 different parameters separated the temperature treatments and two silage corn genotypes into four quadrants (Q) of the PCA. The observation plot showed a clear segregation of five temperature regimes where 25 °C and 20 °C were grouped in the right region of the F1 (Q3 and Q4), whereas 10 °C and 5 °C were observed in the right region of the F1 (Q1 and Q3), 15 °C was grouped in the intermediate region of these groups (Figure 5A). I also observed clear segregation of two silage corn genotypes, Yukon-R was shown in the Q3, whereas A4177G3-RIB in the Q2 (Figure 2.6A). The biplot showed that the silage corn morphological and photosynthetic parameters had a strong positive relationship. However, the biochemical attributes were shown in the opposite region, suggesting that low temperatures enhanced ROS accumulation and antioxidant activities, whereas the seedling growth was reduced. All biochemical attributes were observed in the same quadrant, and ANOVA results showed an increasing trend of these parameters due to temperature drop. Moreover, APX, CAT, SOD, and proline were strongly associated with Yukon-R under 5 °C and 10 °C treatment, indicating Yukon-R has a better chilling tolerance capacity than A4177G3-RIB (Figure 2.6B).

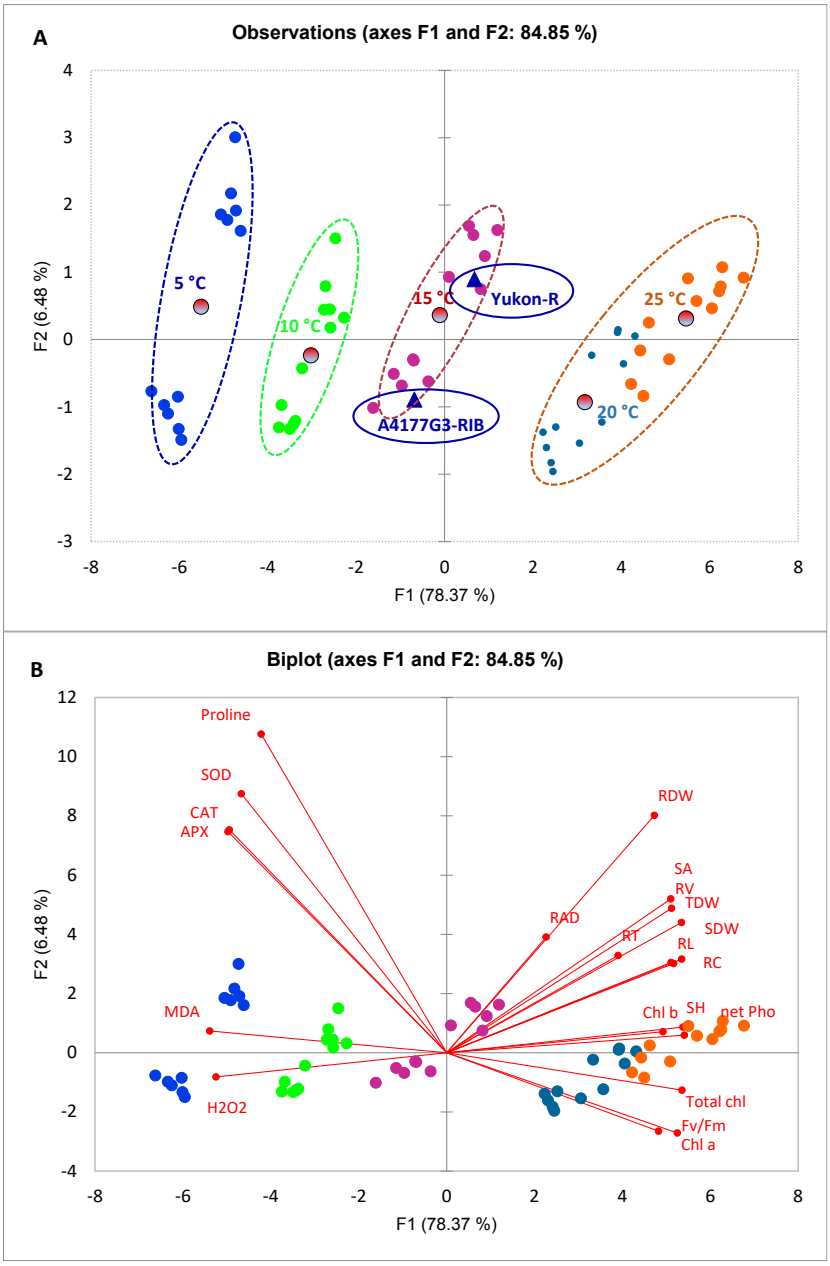


Figure 2.6 Principal component analysis (PCA) observation plot showing separation of different temperature and silage corn genotypes in different quadrants (**A**); PCA biplot showing the association of morphological, physiological, and biochemical attributes of silage corn genotypes grown at different temperatures (**B**). APX: ascorbate peroxidase; CAT: catalase; SOD: superoxide dismutase; Chl a: chlorophyll *a*; Chl b: chlorophyll *b*; H_2O_2: hydrogen peroxide; MDA: malondialdehyde; RDW: root dry weight; RL: root length; RSA: root surface area; RV: root volume; SDW: shoot dry weight; SH: seedling height; total Chl: total chlorophyll.

Pearson's correlation analysis showed negative correlations between silage corn morphological parameters, including RDW, RSA, RV, RL SH, SDW, and MDA, H_2O_2, proline, CAT and APX (Figure 2.7A). Similarly, the photosynthetic parameters (F_v/F_m, *Chl a, Chl b*, total chlorophyll, and photosynthetic rate) were all negatively correlated with the biochemical parameters (Figure 2.7B). For instance, the correlation shows that ROS accumulation (H_2O_2, MDA) and plant growth parameters (morphological and physiological) are strongly negatively correlated (Figure 2.7AB). For the morphological parameters, the highest negative correlation (-0.95) was found between shoot height and MDA content (Figure 2.7A). For the photosynthetic parameters, the highest negative correlation (-0.95) was observed between photosynthetic rate and MDA content as shown in Figure 2.7B.

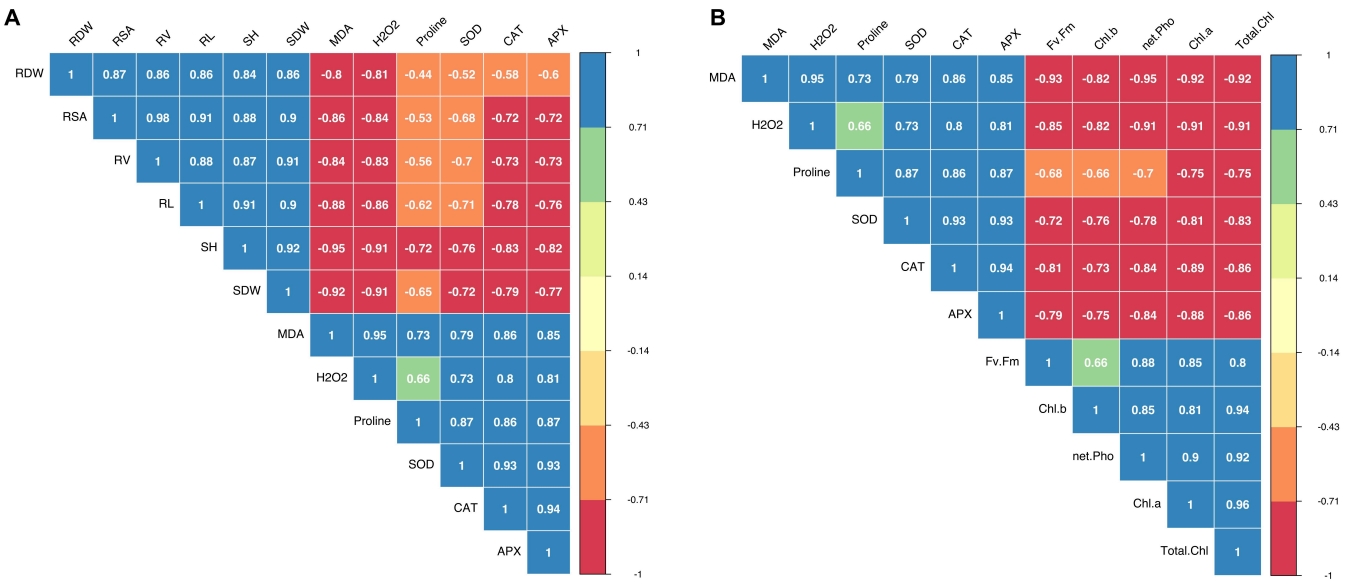


Figure 2.7 The Pearson's correlation matrixes among different parameters in silage corn genotypes when grown at different growth temperatures. Correlation matrix between morphological and biochemical attributes of silage corn (**A**); correlation matrix between silage corn photosynthetic and biochemical parameters (**B**). APX: ascorbate peroxidase; CAT: catalase; SOD: superoxide dismutase; Chl *a*: chlorophyll *a*; Chl *b*: chlorophyll *b*; H_2O_2: hydrogen peroxide; MDA: malondialdehyde; RDW: root dry weight; RL: root length; RSA: root surface area; RV: root volume; SDW: shoot dry weight; SH: seedling height; total Chl: total chlorophyll.

2.4. Discussion

2.4.1. Effects of temp regime on morphological and physiological attributes of silage corn

Low-temperature stress is one of the major abiotic factors which adversely affect the seedling establishment and forage production in boreal agroecosystem (Ali et al. 2019; Kwabiah 2004; Kwabiah et al. 2003). In the present study, low/chilling temperatures (15 °C, 10 °C, and 5 °C) significantly reduced seedling growth, dry weight, and root morphological traits (Figure 2.2) (Table 2.3), total chlorophyll contents, chlorophyll a and b, photosynthesis rate, and F_v/F_m (Figure 2.3A-C) in both genotypes. This reduction in seedling and root morphological traits under low-temperature stress can be attributed to decreased cell division and elongation (Rymen et al. 2007) photosynthetic capacity (Hussain et al. 2016; Sharma et al. 2020) and reduced water, nutrient uptake, and nutrient use efficiency (Ercoli et al. 2004; Grossnickle 2005).

Despite the fact we did not analyze the nutrient uptake, the reduced seedling shoot biomass indicated lower seedling growth which might be associated with lower nutrient and water uptake. Hund et al. (2008) demonstrated that root morphological traits play a key role in determining corn chilling tolerance capacity, especially the primary lateral root length. In the present study, we also observed a significant reduction in silage corn root parameters including root length, surface area, and volume in both cultivars in response to chilling stress. However, genotype Yukon-R maintained higher root morphological parameters than A4177G3-RIB by producing mainly fine roots (Figure 2.2, Table 2.2). These results suggested that a better root system of Yukon-R supports higher biomass production under chilling conditions (Figure 2.1, Table 2.1-3). In addition, reduced root growth affects mineral and water absorption and transportation, which results in lower aboveground biomass (Hong et al. 2017; Ruelland et al. 2009). In our study, we observed 38.7%,

48.4%, 40%, and 46.7% reduced shoot length, shoot dry weight, root dry weight, and total seedling dry weight, respectively at 5°C compared to 25 °C (Table 2.1). The higher biomass production of Yukon-R than A4177G3-RIB might be associated with its higher stress tolerance capacity to perform better under lower growth temperatures of 5 °C or 10 °C.

The chlorophylls are involved in the initial event of photosynthesis, including light absorbing, energy transfer and light energy transducing (Hillier and Babcock 2001; Masuda 2008). Many studies indicated the negative impacts of chilling stress on chlorophyll contents (Farooq et al. 2009; Singh et al. 2012; Turk and Erdal 2015). It is reported that the reduction of chlorophyll content levels can increase the level of energy dissipation, which decreases PS-II efficiency (Demmig-Adams and Adams 2018; Hajihashemi et al. 2018). However, reduced chlorophyll content is not involved in chloroplast development (Hughes and Langdale 2020), which also might improve photosynthetic light use efficiency by increasing light penetration and distribution within the canopy (Slattery et al. 2017). Decrease of chlorophyll contents under chilling stress mainly due to the chlorophyll biosynthesis disruption and chlorophyll degradation. Zhao et al. (2020) reported lower activities of glutamate-1-semialdehyde transaminase, magnesium chelatase, and protochlorophyllide oxidoreductase when rice plants were grown at 12 °C for 48 h during the greening phase. In the present study, we also observed that chilling stress caused a significant reduction in Chl *a*, Chl *b,* and total chlorophyll content (Figure 2.3). The Chl *a*, Chl *b,* and total chlorophyll contents were markedly reduced by 47.2%, 60.5%, 53.4%, respectively when the seedlings were exposed to 5 °C compared to 25 °C (Figure 2.3). Lidon et al. (2001) also reported reduced chlorophyll pigments due to chilling stress. Therefore, the reduced Chl *a*, Chl *b,* and total chlorophyll content in our study might be associated with lower enzyme activities as reported by Zhao et al. (2020).

C_4 plants such as corn are considered efficient in water and nitrogen use compared to C_3 plants. However, their photosynthetic system is strongly affected by chilling temperatures (Long and Spence 2013; Naidu et al. 2003). Kubien et al. (2003) indicated that C_4 plants have less ribulose-1,5-bisphosphate carboxylase (Rubisco) content than C_3 plants in cool climates. Therefore, Rubisco capacity is the key factor limiting the C_4 photosynthesis rate under chilling conditions (Salesse-Smith et al. 2020).

Under the chilling stress, a 60% reduction of the CO_2 assimilation capacity has been reported in the growing plants, thus causing a lower growth rate and grain yield (Long and Spence 2013). Compared to the 25 °C, low-temperature treatments of 15 °C, 10 °C, and 5 °C significantly reduced net photosynthesis rates by 26.4%, 49.1%, and 64.1%, respectively (Figure 2.4A). The decreased photosynthesis rate is attributed to chilling-induced stomal closure and loss of enzyme activities of CO_2 concentration mechanisms (Doubnerová and Ryšlavá 2011; Han et al. 2017). Here we also found that the photosynthesis rate significantly decreased when silage corn seedlings were exposed to lower than 20 °C for 5 days, which showed the high sensitivity of silage corn in response to temperature decrease. Yukon-R had a 10 % higher photosynthesis rate than A4177G3-RIB (Figure 2.4B), which suggests that Yukon-R has a greater potential for forage crop production in cool climates due to its high CO_2 fixation rate.

The F_v/F_m ratio represents the maximal photochemical efficiency of PS-II, which is widely used in detecting the PS-II photoinhibition induced by chilling stress in many plant species (Grzybowski et al. 2019; Han et al. 2017; Ke et al. 2004; Zhang et al. 2021). Chilling stress makes a decline in this ratio mainly from decreasing light absorption in the thylakoid electron transport of PS-II and increasing excitation energy quenching in the light-harvesting antennae (Bilska and Sowiński 2010;

Guidi et al. 2019). Moreover, chilling-induced ROS (H_2O_2 and $O_2^{\cdot-}$) can damage PS-II, and the biosynthesis of D1 protein for PS-II repair during chilling conditions is also inhibited (Gururani et al. 2015; Ruelland et al. 2009). Chlorophyll fluorescence analysis is considered a valid tool for selecting chilling tolerant corn genotypes (Fracheboud et al. 1999; Hund et al. 2008). Recently, Yi et al. (2020) reported that most quantitative trait loci (QTLs) for cold tolerance were associated with the ratio of F_v/F_m in corn. In the present study, we found that low temperature/chilling stress of 5 °C and 10 °C significantly reduced the ratio of *Fv/Fm* in both genotypes (Figure 2.4A), similar results were also reported from previous studies (Urrutia et al. 2021; Zhu et al. 2010). Besides, the interactions between temperature and genotype were significant. The F_v/F_m ratio in Yukon-R was significantly higher than A4177G3-RIB under severe chilling stress conditions of 5 °C and 10 °C (Figure 2.4C), which could be attributed due to higher efficiency of energy transfer and less damage to PS-II in Yukon-R under chilling stress (Allen and Ort 2001). Interestingly, at a mild chilling temperature (15 °C) condition, there was no significant difference in F_v/F_m values between the two genotypes and the decline of *Fv/Fm* was slightly less compared with control (Figure 2.4C). This might be due to silage corn acclimation to the mild chilling stress conditions (Riva-Roveda et al. 2016).

2.4.2. Chilling stress-induced osmotic stress, ROS and redox homeostasis in silage corn seedlings

The measurement of MDA content has been used as a lipid peroxidation biomarker in many studies related to cold stress response (Morsy et al. 2007; Quan et al. 2004; Sun et al. 2020). The over-accumulation of ROS during chilling stress can cause lipid peroxidation which increases membrane leakage and decreases membrane fluidity, which is also considered the main reason for

damaged membrane-localized proteins associated with ion-channels, receptors, and enzymes architecture (Das and Roychoudhury 2014). Polyunsaturated fatty acids, such as linoleic and linolenic acid, are prone to attack by ROS under chilling stress. In addition, MDA strongly affects biomolecules and stress-related gene expression in plant cells (Esterbauer and Cheeseman 1990; Weber et al. 2004). Erdal (2012) reported that the MDA and H_2O_2 contents increased by 27.5% and 22.6%, respectively, under chilling temperatures (10 °C/7 °C day/night) for 3 days. Huang and Guo (2005) indicated that the oxidative stress levels are different between rice cultivars with different chilling sensitivity, and the lower ROS and MDA contents were highly associated with chilling stress tolerance. In the present study, low/chilling temperature stress treatments increased H_2O_2 and MDA contents in silage corn seedlings, interactive effects of temperature and genotype on H_2O_2 and MDA contents were significant (Figure 2.5AB). We have also found that the H_2O_2 and MDA contents of A4177G3-RIB were 35.2% and 16.5%, respectively, higher than Yukon-R at 5 °C. The generation of H_2O_2 occurs in various cellular sites in plant cell, including chloroplast, peroxisome, mitochondria, plasma membrane and in cytoplasm (Hasanuzzaman et al. 2020). Besides, chilling stress limits CO_2 fixation and $NADP^+$ generation, which induces over-reduction of photosynthetic electron transport chain (Gururani et al. 2015; Herath 2018). It results in the accumulation of $O_2^{\bullet-}$ in chloroplast, and H_2O_2 is accumulated by the action of SOD (Sachdev et al. 2021). Chilling stress also can affect the equilibrium in the production and scavenging of H_2O_2, and activities of scavenging enzymes such as APX may be suppressed (Suzuki et al. 2012). Thus, the present study suggests that Yukon-R has a higher chilling stress tolerance capacity due to its lower ROS and MDA accumulations than A4177G3-RIB.

The osmotic potential could be severely affected when plants are exposed to chilling stress conditions, and wilting is one of the visible symptoms caused by chilling temperatures (Farooqi et

al. 2018). Proline is an important osmoprotectant and signaling molecule to avoid chilling injury induced by chilling stress (Duncan and Widholm 1987; Farooq et al. 2009). Proline can prevent protein aggregation during protein refolding, which shows the critical role of proline in stabilizing and enhancing antioxidant enzyme activities under stress conditions (Liang et al. 2013; Samuel et al. 2000). Previous studies also revealed the function of proline in scavenging ROS such as $^1O_2$ (Alia et al. 1997; Matysik et al. 2002; Szabados and Savouré 2010). Moreover, several studies suggested that exogenous application of proline or modification of its responsive genes are effective strategies to improve chilling tolerance (Hayat et al. 2012). In the present study, the interaction between temperature and genotype significantly affects proline content in silage corn leaf tissues. Compared to the non-chilling temperature conditions (20 °C and 25 °C), proline content increased dramatically during chilling stress in silage corn seedlings (Figure 2.5C). Proline contents in Yukon-R at 15 °C, 10 °C, and 5 °C treatments were significantly increased by 84.5%, 104.1%, and 172.1%, respectively (Figure 2.5C). The main role of increased accumulation of free proline probably contributes to increased osmotic potential to avoid chilling-induced dehydration in silage corn seedlings (Hare et al. 1999). Besides, proline may contribute to scavenging of ROS, stabilizing membrane and proteins, as well as regulating the proteins synthesis under chilling stress (Hayat et al. 2012; Mansour 1998). The result also suggests that Yukon-R has superior osmotic regulatory ability in response to chilling response.

Chilling stress contributes to the over-accumulation of ROS in growing plants (Erdal 2012; Suzuki et al. 2012). The increased enzymatic antioxidant activities are vital to minimize ROS toxicity in plant cells under chilling stress conditions. Moreover, transcript levels of antioxidant enzymes have also been found to be up regulated by low temperatures and transgenic overexpression of antioxidant genes can also enhance plant chilling tolerance (Das and Roychoudhury 2014; Sato et

al. 2011; Shafi et al. 2014; Zhou et al. 2017). The PCA results also indicated positive effects of the chilling temperature regime group (10 °C and 5 °C) on antioxidant enzymes (Figure 2.6AB). It's vital to balance the steady-state level between $O_2^{\bullet-}$ and H_2O_2 in plant cells, which could prevent the generation of highly toxic hydroxyl free radical by Harber-Weiss reaction and Fenton reaction under chilling stress conditions (Kehrer 2000; L. Kremer 1999; Mittler 2002). The SOD is ubiquitous in plant cells and acts as the first line in detoxifying ROS, which could catalyze $O_2^{\bullet-}$ to H_2O_2 (Gill and Tuteja 2010b). There are three isozymes of SODs based on their metal cofactors, which include Cu/Zn-SOD (cytosol, peroxisome, and chloroplast), Fe-SOD (chloroplast), and Mn-SOD (mitochondria) (Das and Roychoudhury 2014). Once H_2O_2 was generated, then plants require CAT and APX to metabolize it. The CAT plays an important role in decomposing H_2O_2 to H_2O in the peroxisomes, which is highly expressed and has a fast reaction rate in plants (Das and Roychoudhury 2014; Mhamdi et al. 2010). The APX is involved in the water-water cycle and ascorbate-glutathione cycle in the chloroplast (sAPX) and cytosolic (cAPX), which utilizes AsA to reduce H_2O_2 to H_2O (Apel and Hirt 2004). Compared with CAT, APX is considered an effective H_2O_2 scavenger due to its better affinity for H_2O_2 (Gill and Tuteja 2010a). The antioxidant defense system activation was observed and accompanied by chilling tolerance in many plant species, such as rice (Han et al. 2017), corn (Cao et al. 2019; Hodges et al. 1996), and wheat (Wang et al. 2018). In the present study, our results indicated that the activities of CAT, SOD, and APX were all up-regulated under low/chilling temperature stress, and the interaction of temperature and genotype was significant (Figure 2.5D-F). Compared with A4177G3-RIB, Yukon-R had higher enzymatic activities under severe chilling stress, which is consistent with the lower H_2O_2 and MDA content we found in this genotype (Figure 2.5A-F). Moreover, the higher photosynthesis rate in Yukon-R may be attributed to higher SOD and APX activities in chloroplast under chilling stress (Gururani

et al. 2015; Maruta et al. 2009). The up-regulation activity of CAT, SOD and APX is considered an important strategy to alleviate chilling-induced oxidative stress, and some ROS molecules such as H_2O_2 also induce antioxidant enzyme-related gene expression (Awasthi et al. 2015; Dvořák et al. 2021; Prasad et al. 1994; Zhang et al. 2009). The balance of SOD, CAT and APX is crucial for controlling the H_2O_2 level in a cell (Apel and Hirt 2004). These antioxidant enzymes were positively correlated to corn chilling stress tolerance and were reported to be helpful in screening for chilling tolerance in different corn varieties (Hodges et al. 1996; 1997a; b).

2.5. Conclusion

In summary, the results from this study showed that low/chilling temperature stress induces significant morphological, physiological, and biochemical changes in silage corn seedlings at the early growth stage. Root growth and distribution such as root weight, length, surface area, and root volume were greatly reduced under stressed conditions, which ultimately inhibited silage corn aboveground growth. Moreover, chilling temperatures, particularly 10 °C and 5 °C, triggered higher ROS accumulation and lower photosynthetic capacity than non-chilling conditions. Silage corn genotypes exhibited differential tolerance capacity in response to chilling and non-chilling stress. Genotype Yukon-R produced seedlings with higher shoot growth/biomass and lower H_2O_2 and MDA contents than A4177G3-RIB under chilling stress. The higher photosynthesis, proline content, and antioxidant enzymatic activities contributed to improved seedling biomass and chilling tolerance in Yukon-R. Moreover, over-accumulation of ROS and lipid peroxidation at the early growth stage contributed to significant reductions in the growth and root morphological traits of silage corn seedlings under chilling stress conditions. This study provides useful information on potential silage corn genotypes with cold tolerant traits that may be suitable for cultivation in

boreal climates. Further research is needed to find the key regulators involved in silage corn chilling stress response using appropriate approaches such as lipidomic and transcriptomics analyses.

2.6. References

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Chapter 3

Lipidomics remodelling: a strategy to enhance cold stress tolerance in silage corn

Abstract

Phospholipids are a major component of plant biological membranes, which are involved in plant growth and developmental processes. Several recent studies have shown the importance of phospholipids in plant development and cold stress acclimation. However, there is a paucity of information available concerning how the phospholipidome metabolism contributes to cold stress tolerance in silage corn ideally suited for cultivation in boreal climate. Herein, the leaf and root membrane lipidome was assessed using C30 reverse phase chromatography and high-resolution accurate mass, tandem mass spectrometry (C30 RPLC- HRAM-MS/MS) to determine the changes in phospholipid metabolism under different temperature regimes (25 °C, 20 °C, 15 °C, 10 °C and 5 °C) in two silage corn genotypes (Yukon-R and A4177G3-RIB). Phospholipid composition of silage corn seedling leaves and roots was significantly affected by growth temperatures. The results showed that cold temperature stress significantly ($p < 0.001$) reduced phosphatidylcholine (PC), and phosphatidylglycerol (PG), but increased the levels of phosphatidylethanolamine (PE) in silage corn leaf and root. Furthermore, cold stress increased the anabolism of phosphatidic acid (PA) in silage corn leaf and roots under cold stress conditions which occurred concomitantly with elevated expression of the PA biosynthesis genes related to upregulation of the phospholipase D and phospholipase C-diacylglycerol kinase pathways. Yukon-R had significantly higher levels of PA in leaf ($p < 0.001$) and root ($p < 0.05$) than A4177G3-RIB following exposure to cold temperature stress which suggests possibly enhanced cold tolerance in Yukon-R compared to

A4177G3-RIB. In summary, maintenance of membrane integrity and increased PA content could be adaptive strategies in silage corn at the early growth stage. Our findings would be helpful in selecting or developing silage corn genotypes ideally suited for cultivation in boreal climate.

Keywords: cold stress, lipidomic, phospholipid, phosphatidic acid, biosynthesis, silage corn

3.1. Introduction

Cold stress is one of the major abiotic stresses that limits forage biomass productions of silage corn in the boreal climate (Kwabiah et al. 2003; Nadeem et al. 2020). It is reported that cold temperatures negatively affect plant growth by disrupting the physiological, biochemical and molecular processes, such as photosynthesis, protein synthesis and RNA folding (Farooq et al. 2009). The plant is impacted by cold stress particularly in the early growth stages, and tolerance to cold stress is necessary for a plant to survive in a cool climate such as that of the boreal agroecosystem which sees an average temperature of 5 °C to 12 °C during the growing season (Ali et al. 2019). Therefore, understanding the plant's signal transduction and tolerance mechanisms to cold stress are vital for developing cold resistant crops in the future (Burnett and Kromdijk 2022).

Cellular membranes are dynamic structures that can help plants to acclimatise and adapt to environmental changes, such as extremely low temperatures by altering the membrane lipid composition and unsaturation levels of fatty acids (Barrero-Sicilia et al. 2017; Lamers et al. 2020). Besides, plasma membranes play an indispensable role in plant signal recognition and transduction by hosting receptors and mediating protein-lipid interactions (de Jong and Munnik 2021). Cold stress reduces the fluid state of cellular membranes, which changes the physical conformation and

play a negative role in stabilizing membrane lipids and proteins (Barrero-Sicilia et al. 2017; Ding et al. 2019). Remodeling the lipid composition of the plasma membrane to maintain membrane stability and functions under cold stress is a recognized strategy adopted by plants (Barrero-Sicilia et al. 2017; Moellering et al. 2010; Wu et al. 2020). For instance, fatty acid desaturation level of lipid membrane is positively correlated with plant cold stress tolerance; therefore, the unsaturation of lipid is helpful in preventing plant cold injury (Chen and Thelen 2013).

Glycerolipids, sphingolipids and sterols are three major classes of lipids in biological membranes of plants. Phospholipids are subgroups of glycerolipids and are emerging as potent second messengers in plant stress response and tolerance mechanisms (Adigun et al. 2021; Barrero-Sicilia et al. 2017; Meijer and Munnik 2003). Recently published studies have shown that cold stress can modify membrane phospholipid profiles which play critical roles in mediating plasma membrane integrity and signal transduction in plant cells (Gu et al. 2018; Nadeem et al. 2019a; Zhao et al. 2021). For instance, during stress conditions, changes in two dominant structural phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), are usually related to phospholipase activities (Nadeem et al. 2020; Wu et al. 2020). The PC is a major bilayer-forming lipid in plasma membranes, but not PE (Hull et al. 2004); therefore, a ratio of PC/PE could be used as a potential biomarker of plasma membrane integrity (Barrero-Sicilia et al. 2017; Wu et al. 2020). Phosphatidylglycerol (PG) is the only detectable phospholipid in the thylakoid membranes, which is believed to play a critical role in primary photosynthetic processes, such as electron transport and light reactions (Callahan et al. 2022, Guo et al. 2019; Hagio et al. 2002; Wada and Mizusawa 2009). Therefore, monitoring PG content is important for photosynthetic processes which are impaired by cold temperatures. The *phosphatidylglycerol biosynthesis-deficient* (*pgp1*) mutants showed impaired photosynthesis in *Arabidopsis*, suggesting that PG might be necessary for the

stabilization of the chloroplast and photosynthetic machinery (Xu et al. 2002). It is reported that PG 16:0-16:0 and PG16:0-16:1 are two molecular species that contribute significantly to cold sensitivity in plants (Murata 1983; Wada and Murata 2007). Several studies revealed that the unsaturation levels of PG were negatively correlated with photoinhibition under cold stress conditions (Chen et al. 2013; Ivanov et al. 2012; Roughan 1985). Increased levels of saturated fatty acids in PG led to more sensitivity to photoinhibition, reduced plant growth and damage to flower inflorescences in tobacco plants exposed to cold temperatures (Moon et al. 1995; Sakamoto et al. 2004).

There are some less abundant signaling phospholipids, including phosphatidic acid (PA), phosphatidylserine (PS) and phosphatidylinositol (PI) that are involved in lipid turnover and stress signal transduction (Hong et al. 2016; Perlikowski et al. 2016; Yu et al. 2019). However, rapid accumulation of signaling lipids and turnover was observed in growing plants in response to external stress conditions and may explain in part, how the lower composition of these lipids confers potent or successful stress acclimation (Hong et al. 2016; Hou et al. 2016). These signaling lipids are able to change cell metabolism *via* activating membrane-localized target protein (van Leeuwen et al. 2004). PA, a typical signaling phospholipid, has been identified as a secondary messenger in mediating cellular stress signal transduction in higher plants, including salt, drought, and temperature stresses (Arisz et al. 2013; Yao and Xue 2018). PA accounts for only ~1% of total lipid content, but its level increases significantly in response to cold stimuli (Li et al. 2004; Vilchez et al. 2021; Zhao et al. 2021). Du et al. (2010) reported that membrane-associated PA can bind acyl-coenzyme A-binding protein 1 (ACBP1) during cold acclimation in *Arabidopsis*. Therefore, the PA biosynthesis and the enzymes (phospholipases and kinases) involved in this process are crucial for plant adaptation to cold temperature conditions.

In response to cold stress, two PA biosynthesis pathways are activated; the phospholipase-D (PLD) pathway and the phospholipase-C (PLC) - diacylglycerol kinase (DGK) pathway (Figure 1.3) (Hong et al. 2016). The phospholipase D pathway is one of the major pathways that hydrolyze common membrane phospholipids in various plants physiological processes, including stress response, root growth and programmed cell death (Wang 2005). The structural phospholipids are hydrolyzed by PLD to produce PA (Hong et al. 2010). The individual PLDs have unique cellular functions in plant associated with PA signaling activation in response to abiotic stress (Wang 2004). The PLC-DGK pathway and its derived PA are very responsive to plant stress conditions (Arisz et al. 2013; Testerink and Munnik 2005; 2011). According to the substrate preferences and functions in plants, PLC can be divided into non-specific PLC (NPC) and phosphoinositide-specific PLC (PI-PLC) (Wang 2001). Firstly, in the PLC-DGK pathway, phospholipids are hydrolyzed by PLC into DAG and a phosphorylated head group. Then, PA is generated by further phosphorylating DAG by DGK (Arisz et al. 2009). Several studies revealed that PLC and DGK members are involved in abiotic stresses-triggered signal transduction (Arisz et al. 2009), and are activated by low temperatures in crops such as soybean, maize, rice, chickpea, and wheat (Sagar et al. 2020; Singh et al. 2013; Wang et al. 2015; Wang et al. 2020; Zhu et al. 2021).

Ruelland et al. (2002) reported that both pathways were activated in *Arabidopsis* exposed to 0 °C or 10 °C temperatures treatment. However, rapid cold-induced PA biosynthesis (within 2 to 5 min) occurred through the PLC-DGK pathway rather than the PLD pathway (Arisz et al. 2013; Delage et al. 2012). Although both pathways can generate PA, it is believed that the activation of the PLC-DGK pathway is functionally different from PLD in plant cold response (Wang et al. 2006). Individual PLD, PLC, and DGK isoforms have unique cellular functions in stress response due to their domain structures and biochemical properties. For instance, PLDα, β, γ, δ, and ϵ isoforms

have the C2 domain near the N-terminal, which require Ca^{2+} for enzyme activation, but PLDζs are Ca^{2+}- independent due to the lack of the C2 domain (Qin and Wang 2002). In addition, the hydrolyzing substrate preferences of each PLD isoform are also different (Li and Wang 2019). Therefore, the expression patterns and specific roles of PA synthesis-related genes involved in cold response and tolerance need to be investigated to reconcile this issue in order to better understand their contributions to successful cold stress tolerance (Wang 2005).

In *Arabidopsis*, two isoenzymes from the PLD family, PLDα1 and PLDδ, play distinct roles in mediating cold response. PLDδ is a plasma membrane-localized enzyme (Wang and Wang 2001), and transgenic analysis showed that 20% PA was produced by PLDδ, which helped *Arabidopsis* in cold acclimation during exposure to freezing temperature stress (Katagiri et al. 2001; Li et al. 2004). In contrast, PLDα1 showed a negative impact on plant cold tolerance, PLDα1-deficient *Arabidopsis* displayed lower ion leakage and higher survival rate in both acclimated and non-acclimated plants exposed to freezing temperatures (Rajashekar et al. 2006; Welti et al. 2002). PLC-DGK pathway has been reported to occur upstream of CBF transcription in cold response signaling, which requires Ca^{2+} for activation (Arisz et al. 2013; Lee et al. 2005; Sanchez and Chua 2001; Shinozaki et al. 2003). Several genes encoding *PI-PLC* and *DGK* were upregulated in plant species under cold stress (Gu et al. 2018; Singh et al. 2013; Tripathy et al. 2012; Zhai et al. 2005; Zhang et al. 2018). For example, Zhu et al. (2021) demonstrated that the transcriptional levels of *ZmPI-PLC3a*, *ZmPI-PLC3b* and *ZmNPC1a* were increased after 30 min under 4°C treatment in corn plants. Moreover, several genes from the DGK gene family, such as *ZmDGK2* and *ZmDGK5* were also significantly upregulated (Gu et al. 2018).

Phospholipid metabolism is involved in plant cold stress response, including stabilization of membrane structures, photosynthetic process, and signal transduction. Therefore, changes in phospholipid metabolism will help to better understand the acclimatization strategy used by silage corn exposed to cold stress conditions during the early growth stages. Silage corn (*Zea mays* L.) belongs to tropical C4 plant species, which is adapted to dry and high temperature conditions. Silage corn is highly palatable, digestible, and easy to ensile due to the high contents of soluble sugar. The boreal agroecosystem is characterized by cold stress (10 °C to 12 °C) during early growth stages. This imposes an array of detrimental effects on silage corn growth and productivity when cultivated in this environment. Besides, increasing evidence is now showing the positive role of PA accumulation in alleviating cold stress effects (Arisz et al. 2013; Vilchez et al. 2021; Zhang et al. 2020). Previous studies conducted by Nadeem et al. (2019b) demonstrated that silage corn genotypes cultivated under field conditions in boreal climate had elevated levels of root PA at the black layer stage, which was positively correlated with superior agronomic performance (increased plant height and biomass production). However, a comprehensive analysis of phospholipid metabolism, particularly PA accumulation in both leaf and root tissues at the early growth stage of silage corn under cold and non-cold temperature stress is needed to better understand how these silage corn genotypes successfully circumvent cold temperature stress in boreal climate to adapt and achieve superior agronomic performance. We hypothesized that cold stress remodels phospholipid metabolism, including PA biosynthesis-related genes in mediating cold stress tolerance. To test the hypothesis, the current study was designed to achieve the following specific objectives:

1. to investigate the effects of cold and non-cold temperature stress on phospholipid metabolism including PA in two silage corn genotypes at the early growth stage.

2. to determine the expression of PA biosynthesis-related genes in mediating stress tolerance under cold and non-cold temperature stress conditions.

3.2. Materials and Methods

3.2.1. Experimental design, plant material and growth conditions

Two silage corn genotypes (Yukon-R and A4177G3-RIB) were selected for this study based on our previous research work (Ali et al. 2019; Nadeem et al. 2019b). For the detailed experimental design, plant growing conditions and different temperature treatments, please refer to section **2.2.1** in **Chapter 2**.

3.2.2. Membrane lipid extraction and lipidomic analysis

3.2.2.1. Chemicals and reagents

The high-performance liquid chromatography (HPLC) grade acetonitrile, chloroform and methanol were purchased from Fisher Scientific (Mississauga, ON, Canada); deionized water was obtained from Barnstead Nanopure Purification System (Thermo Scientific, ON, Canada). The HPLC grade acetic acid, formic acid, ammonium formate and ammonium acetate were purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.2.2.2 Leaf and root of silage corn lipid extraction

Plant leaf and root samples were harvested after imposing 5-days cold and non-cold temperature stress to determine lipidome analysis. Lipid extraction was done following the method of Shiva et al. (2018) with slight modification. Briefly, the fresh plant sample (~20 mg for leaf or ~50 mg for root) was cut and transferred into a pre-weighed 4 mL vial having polytetrafluoroethylene (PTFE) lined cap (VWR, Mississauga, Canada) and incubated in 0.8 mL hot isopropanol (75 °C) with 0.01%

BHT for 15 min to inactivate phospholipase activity (Wu et al. 2020). Then, a 2.4 mL chloroform: methanol: water mixture (30/41.5/3.5, v/v/v) was added into the vials, thus making a final solvent mixture with chloroform: isopropanol: methanol: water in the ratio of 30/25/41.5/3.5 (v/v/v/v). Extracts were shaken at 100 rpm on an orbital shaker for 24 h and transferred to a new 4 mL vial. The samples were then stored at -80°C for further analysis.

3.2.2.3 Lipids analyses by ultra-high performance liquid chromatography and C30 reverse phase column chromatography coupled to heated electrospray ionization high resolution accurate mass tandem mass spectrometry (UHPLC-C30-RPLC)

Lipids present in silage corn leaves or roots were separated on an Accucore C30 reverse phase column (150 × 2 mm I.D., particle size: 2.6 μm, pore diameter: 150 Å). The mobile phase system was prepared using solvent A and solvent B consisting of acetonitrile: H_2O (40:60 v/v) and isopropanol: acetonitrile: H_2O (90:10:1, v/v/v), respectively, and both solvent A and B contained 0.1% formic acid and 10 mM ammonium formate. The separation was carried out at 30 °C (column temperature) with a flow rate of 0.2 mL/min, and 10 μL of the samples suspended in chloroform: methanol (1:1 v/v) were injected into the machine. The following system gradient for lipid classes and molecular species separation was employed according to the methods of Pham et al. (2019). Briefly, 30% solvent B was first applied for 3 min; then solvent B increased to 43% over 5 min. Next, the gradient was increased in 1 min to 50% B, then to 90% B over 9 min followed by an increase to 99% B over 8 min and finally kept at 99% B for 4 min. Before each new injection, the column was re-equilibrated to 70% solvent A (initial conditions) for 5 min.

A Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an automated Dionex UltiMate 3000 UHPLC system controlled by both X-Calibur 4.0

and Chromeleon software, respectively, were used for lipid analyses in this study (Nadeem et al. 2019b; Pham et al. 2019). The parameters for the Q-Exactive Orbitrap mass spectrometer were set as follows: sheath gas: 40, auxiliary gas: 2, ion spray voltage: 3.2 kV, capillary temperature: 300 °C; S-lens RF: 30 V; mass range: 200–2000 *m/z*; full scan mode at a resolution of 70,000 m/z; top-20 data dependent MS/MS at a resolution of 35,000 *m/z* and collision energy set at 35 (arbitrary unit); injection time: 50 min; isolation window: 1 *m/z*; automatic gain control target: 1e5. Both ESI negative and positive calibration solutions were used for the instrument external calibration to 1 ppm.

3.2.3 RNA isolation, cDNA synthesis and quantitative RT-PCR analysis

The leaf tissues (100 mg) were immediately harvested in liquid nitrogen (N_2) after different temperature treatments for 24 h, and then stored at -80 °C until further extraction. TRIzol™ Plus RNA purification kit (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from silage corn leaf tissue according to the manufacturer's protocol. Genomic DNA was removed by Turbo DNA-*free*™ Kit (Invitrogen, Carlsbad, CA, USA), followed by the quantification by NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA). Afterward, 2 µg total RNA was reverse-transcribed for synthesis first strand cDNA using High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Carlsbad, CA, USA).

The qRT-PCR was performed using PowerUp™ SYBR™ Green Master (Applied Biosystems, Carlsbad, CA, USA) and specific primers in 10 µL reactions and conducted on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR cycling conditions were set up as follows: uracil-DNA glycosylase (UDG) activation at 50 °C for 2 min, followed by denaturation at 95 °C for 2 min and 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C

for 15 s and extension at 72 °C for 60 s. Melting curve analysis was also performed after the real-time PCR to confirm the specificity of the primers. The relative expression level was normalized by *ZmGAPDH* gene from corn and calculated through the delta-delta Ct ($2^{-\Delta\Delta Ct}$) method (Schmittgen and Livak 2008). Three biological replications and three technical replications were applied in this gene expression analysis. Primer pairs used are listed in Table 3.1.

Table 3.1 List of primers used in this study for qRT-PCR analysis.

Gene name	Forward primer (5' → 3')	Reverse primer (5' → 3')	Maize GDB locus	Reference
ZmPLDα1	CGCACAAGCTCTCGAAGCCAA	TGATGATGTACTCGTCGTCCACTAT	GRMZM2G054559	
ZmPLDα2	GCCAAAATGATGATAGTGGACGA	TCCAGCATCCCCAGGTGT	GRMZM2G061969	
ZmPLDδ1	TGGCGGCAAGATTCTGGGAG	TGTGGTTGGATCTCCATGAGCC	GRMZM2G108912	Chen et al. (2017)
ZmPLDδ2	ACAGATAAAAGTGCAGCGGCCTTAG	TGGTATGCACCCATTGCGATCT	GRMZM2G140811	
ZmPLC1	CATCCTCGCCATGCTCT	AATGACTCTAACACCTCCCTG	GRMZM5G889467	Wang et al. (2008)
ZmDGK1	GACACTTACCTCCCTCAGTCCAT	CCTGGCTATGATGAGAAACCTCT	GRMZM2G076911	
ZmDGK2	TAGCAAATGAGCCGTGCAGGTCC	TGAGCAATGTCAATCTCGTCAGGAATC	GRMZM2G094452	
ZmGAPDH	CCATCACTGCCACACAGAAAAC	AGGAACACGGAAGGACATACCAG	GRMZM2G046804	Lin et al. (2014)

3.2.4 Statistical analyses

This study was performed two times independently to validate the results, and the data set for two experiments was pooled and analyzed. The level of lipids was expressed by a semi-quantitation using nanomole (nmol%) in the silage corn samples. The nmol% was calculated by dividing the nmol area of each lipid molecular species by the sum of the same lipid class. The Shapiro-Wilk test was employed to check the normality of data before further statistical analysis was done. The XLSTAT program (Permium 2020, Addinsoft Inc, NY, USA) was used for one-way and two-way analysis of variance (ANOVA) and means were compared using Fisher's Least Significant test ($p \leq 0.05$) when alpha was less than 5% for the model. All data in this study were expressed as means ± standard error (SE). Figures were prepared using GraphPad Prism (version 8.4.3, GraphPad Software, CA, USA). Principal component analysis (PCA) and its visualization were performed using the XLSTAT program to determine the relationships between temperatures, genotypes, and phospholipid abundance. The R statistic software was used to conduct the Pearson's correlation analysis (R Core Team 2021).

3.3. Results

3.3.1. Assessment of silage corn leaves and roots lipidome in response to different temperature regimes

The lipidome in seedling leaf and root of two silage corn genotypes were evaluated to assess the changes in lipid metabolism in response to different temperatures exposure at V3 stage (Table 3.2 - 3.3). Overall, four classes of lipids were observed in leaf and root including phospholipids, glycolipids, sphingolipids and phytosterols, irrespective of temperatures and genotypes (Table 3.2 – 3.3). There were 21 lipid classes in total including ten phospholipids [PA, PC, PE PG, PI, PS,

lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) and lysophosphatidylglycerol (LPG) and cardiolipin (CL)]; two glycerolipids [diacylglycerol (DG) and triacylglycerol (TG)]; three sphingolipids [sphingomyelin (SM), hexosylceramide (HexCer) and ceramide (Cer)] and seven phytosterols [beta sitosterol (SiE), stigmasterol ester (StE), campesterol ester (CmE), acylated hexosyl stigmasterol ester (AcHexStE), acylated hexosyl betasitosterol ester (AcHexSiE) and acylated hexosyl campesterol ester (AcHexCmE)] as shown in Table 3.2 and Table 3.3.

Significantly (p<0.05) higher phospholipids were observed in seedling leaves and roots compared to other lipid classes, irrespective of silage corn genotypes or temperature regimes (Table 3.2). Overall, the total lipid profile varied in order of phospholipids > glycerolipids > sphingolipids > phytosterols in leaves or roots as shown in Table 3.1 and Table 3.2. Phospholipids represent 66.02±1.43% to 69.81±0.43% in silage corn leaf, and 68.23±2.99% to 75.56±1.10% in silage corn root (Table 3.1 and Table 3.2). Glycerolipids presented the second highest abundance ranging from 20.32±0.36% to 24.59±0.98% in leaf and 19.22±0.76% to 25.60±1.92% in the root, respectively (Table 3.2 and 3.3). However, sphingolipids and phytosterols were relatively low as shown in Table 3.2 and Table 3.3.

Table 3.2 Effects of cold temperature regimes on the leaf lipid profile of two silage corn genotypes when grown under controlled environmental conditions.

Lipid classes	Lipid Sub-classes	Relative abundance (nmol%)						
		25°C	20°C	15°C	10°C	5°C	Yukon-R	A4177G3-RIB
Phospholipids	PC	44.51±0.39	41.61±0.34	42.65±0.59	40.03±0.31	36.57±0.67	40.91±0.47	41.21±0.58
	PE	10.65±0.20	10.32±0.20	11.31±0.40	12.84±0.19	14.15±0.82	11.87±0.28	11.85±0.46
	PG	7.16±0.17	6.62±0.25	5.96±0.16	4.78±0.17	4.11±0.24	5.97±0.21	5.48±0.26
	PA	2.54±0.22	2.91±0.26	5.08±0.28	6.36±0.28	7.79±0.25	5.45±0.44	4.44±0.35
	PI	1.65±0.13	1.68±0.14	1.09±0.11	1.21±0.07	1.57±0.10	1.44±0.09	1.45±0.07
	PS	1.24±0.18	0.79±0.05	0.62±0.05	0.55±0.03	0.70±0.04	0.86±0.09	0.70±0.03
	LPC	0.44±0.06	0.30±0.06	0.78±0.06	0.85±0.03	1.14±0.09	0.69±0.08	0.71±0.06
	LPE	0.07±0.007	0.03±0.004	0.02±0.01	0.004±0.002	0.05±0.001	0.04±0.005	0.03±0.006
	LPG	0.04±0.006	0.02±0.003	0.04±0.01	0.02±0.003	0.04±0.006	0.04±0.004	0.02±0.004
	CL	0.38±0.03	0.40±0.04	0.27±0.03	0.28±0.55	0.34±0.03	0.31±0.02	0.35±0.03
Glycerolipids	DG	10.08±0.50	11.18±1.00	9.02±0.97	10.57±0.95	11.59±1.18	10.29±0.66	10.61±0.53
	TG	10.24±0.37	12.75±0.77	12.57±0.87	12.03±0.87	13.00±0.92	12.09±0.48	12.05±0.52
Sphingolipids	Cer	1.49±0.15	1.75±0.16	1.57±0.12	1.83±0.48	1.78±0.36	1.49±0.09	1.88±0.03
	HexCer	4.62±0.47	4.12±0.56	4.89±0.52	4.45±0.49	2.55±0.58	4.27±0.34	4.18±0.32
	SM	0.52±0.08	0.95±0.15	0.66±0.19	0.69±0.46	0.66±0.10	0.57±0.06	0.82±0.11
Phytosterols	AcHexCmE	0.84±0.06	0.95±0.12	0.74±0.09	0.76±0.08	0.82±0.05	0.71±0.04	0.93±0.05
	AcHexSiE	0.88±0.06	0.91±0.16	0.69±0.09	0.77±0.11	0.84±0.08	0.84±0.04	0.80±0.05
	AcHexStE	0.88±0.10	0.91±0.09	0.70±0.07	0.72±0.10	0.96±0.07	0.75±0.05	0.92±0.06
	CmE	0.63±0.08	0.53±0.06	0.45±0.06	0.38±0.09	0.44±0.05	0.45±0.03	0.52±0.06
	SiE	0.77±0.07	0.88±0.14	0.63±0.12	0.59±0.11	0.56±0.08	0.68±0.05	0.69±0.08
	StE	0.36±0.03	0.40±0.04	0.27±0.03	0.29±0.06	0.33±0.03	0.31±0.02	0.36±0.03
Total		**100**	**100**	**100**	**100**	**100**	**100**	**100**
Phospholipids *		**68.70±0.43 a**	**64.67±1.43 b**	**67.81±1.06 a**	**66.93±1.41 ab**	**66.46±1.02 ab**	**67.58±0.63**	**66.24±0.82**
Glycerolipids *		**20.32±0.36 b**	**23.93±1.06 a**	**21.59±1.08 ab**	**22.60±1.37 ab**	**24.59±0.98 a**	**22.38±0.60**	**22.66±0.76**
Sphingolipids *		**6.64±0.44 ab**	**6.82±0.50 ab**	**7.12±0.55 a**	**6.97±0.59 a**	**4.99±0.57 b**	**6.34±0.29**	**6.88±0.39**
Phytosterols *		**4.35±0.26 ab**	**4.58±0.40 a**	**3.48±0.34 b**	**3.51±0.52 b**	**3.96±0.27 ab**	**3.74±0.13**	**4.22±0.31**

* represents significant differences at alpha 0.05. The values (nanomole%) present here are means ± standard errors (n=12 for temperatures, n=30 for genotypes). Different letters within each column indicate significant differences among five growth temperatures or two silage corn genotypes according to Fishers' Least Significant test (p=0.05). The lipids detected were: phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylglycerol inositol (PI), phosphatidylglycerol serine (PS), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylglycerol (LPG), triacylglycerol (TG), diacylglycerol (DG), sphingomyelin (SM), beta sitosterol (SiE), stigmasterol ester (StE), hexosylceramide (HexCer), ceramide (Cer), campesterol ester (CmE), acylated hexosyl stigmasterol ester (AcHexStE), acylated hexosyl betasitosterol ester (AcHexSiE), cardiolipin (CL), and acylated hexosyl campesterol ester (AcHexCmE).

Table 3.3 Effects of cold temperature regimes on the root lipid profile of two silage corn genotypes when grown under controlled environmental conditions

Lipid classes	Lipid Sub-classes	Relative abundance (nmol%)						
		25°C	20°C	15°C	10°C	5°C	Yukon-R	A4177G3-RIB
Phospholipids	PC	45.30±0.29	45.19±0.30	41.60±0.57	45.11±0.94	38.57±0.56	42.96±0.66	43.81±0.62
	PE	10.53±0.25	11.22±0.28	12.69±0.44	14.43±0.68	14.37±0.71	12.54±0.33	12.81±0.42
	PG	3.69±0.24	3.09±0.18	3.38±0.21	3.05±0.15	2.77±0.13	3.51±0.14	2.92±0.11
	PA	2.80±0.25	3.16±0.22	4.28±0.13	6.75±0.63	8.22±0.57	5.44±0.50	4.59±0.34
	PI	2.36±0.15	2.20±0.19	2.23±0.18	2.24±0.19	1.98±0.23	2.06±0.12	2.37±0.11
	PS	1.99±0.08	1.93±0.32	2.10±0.20	1.84±0.17	2.10±0.25	1.91±0.12	2.10±0.15
	LPC	0.18±0.04	0.25±0.07	0.31±0.08	0.85±0.02	0.95±0.03	0.53±0.07	0.48±0.06
	LPE	0.01±0.005	0.01±0.002	0.01±0.002	0.01±0.002	0.04±0.01	0.03±0.01	0.005±0.0007
	LPG	0.001±0.0001	0.001±0.0002	0.001±0.0002	0.001±0.0002	0.004±0.001	0.003±0.001	0.0005±0.0001
	CL	0.24±0.08	0.13±0.03	0.11±0.04	0.13±0.006	0.05±0.01	0.10±0.03	0.08±0.02
Glycerolipids	DG	9.67±0.56	11.22±0.58	12.08±1.10	9.64±0.69	11.02±0.59	11.25±0.49	10.32±0.46
	TG	10.41±0.30	12.12±0.56	13.52±1.20	9.58±0.37	9.54±0.66	10.92±0.39	11.48±0.57
Sphingolipids	Cer	1.90±0.08	1.72±0.23	1.63±0.24	1.29±0.07	2.57±0.24	1.72±0.13	1.93±0.16
	HexCer	7.56±0.59	5.83±0.64	4.61±0.63	3.88±0.37	5.76±0.48	5.47±0.40	5.59±0.43
	SM	0.01±0.003	0.01±0.002	0.008±0.001	0.009±0.001	0.01±0.002	0.01±0.001	0.01±0.002
Phytosterols	AcHexCmE	0.33±0.05	0.25±0.04	0.25±0.05	0.27±0.02	1.17±0.09	0.43±0.07	0.48±0.08
	AcHexSiE	0.19±0.06	0.15±0.03	0.17±0.04	0.18±0.005	0.33±0.04	0.20±0.03	0.16±0.02
	AcHexStE	0.04±0.006	0.04±0.005	0.05±0.006	0.06±0.005	0.41±0.05	0.10±0.02	0.14±0.04
	CmE	0.38±0.08	0.60±0.15	0.39±0.15	0.29±0.007	0.09±0.01	0.32±0.07	0.30±0.07
	SiE	0.60±0.16	0.87±0.23	0.58±0.21	0.49±0.009	0.07±0.01	0.47±0.11	0.42±0.11
	StE	0.005±0.002	0.004±0.001	0.003±0.001	0.002±0.0005	0.002±0.0005	0.003±0.005	0.004±0.0007
Total		**100**	**100**	**100**	**100**	**100**	**100**	**100**
Phospholipids *		**68.90±1.50 b**	**67.18±1.81 b**	**66.71±2.99 b**	**74.40±1.10 a**	**69.04±1.33 ab**	**69.07±1.17**	**69.17±1.35**
Glycerolipids *		**20.08±0.67 c**	**23.34±0.84 ab**	**25.60±1.92 a**	**19.22±0.76 c**	**20.56±0.72 bc**	**22.16±0.70**	**21.81±0.59**
Sphingolipids *		**9.47±0.74 a**	**7.56±0.84 ab**	**6.24±0.85 bc**	**5.18±0.42 c**	**8.33±0.68 ab**	**7.19±0.49**	**7.52±0.56**
Phytosterols *		**1.54±0.34 a**	**1.92±0.47 a**	**1.45±0.47 ab**	**1.20±0.37 b**	**2.07±0.15 a**	**1.57±0.24**	**1.50±0.23**

* represents significant differences at alpha 0.05. The values (nanomole%) present here are means ± standard errors (n=12 for temperatures, n=30 for genotypes). Different letters within each column indicate significant differences among five growth temperatures or two silage corn genotypes according to Fishers' Least Significant test (p=0.05). The lipids detected were: phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylglycerol inositol (PI), phosphatidylglycerol serine (PS), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylglycerol (LPG), triacylglycerol (TG), diacylglycerol (DG), sphingomyelin (SM), beta sitosterol (SiE), stigmasterol ester (StE), hexosylceramide (HexCer), ceramide (Cer), campesterol ester (CmE), acylated hexosyl stigmasterol ester (AcHexStE), acylated hexosyl betasitosterol ester (AcHexSiE), cardiolipin (CL), and acylated hexosyl campesterol ester (AcHexCmE).

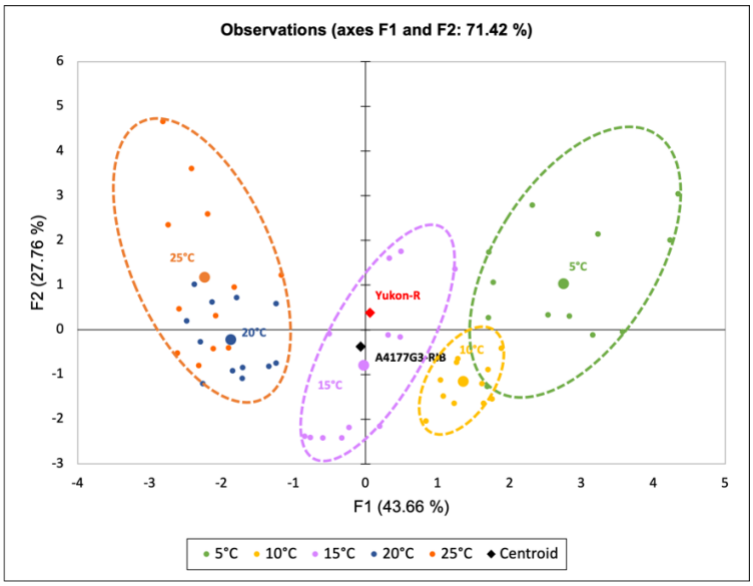
3.3.2. Remodelling of silage corn leaf and root membrane phospholipidome in response to cold temperature exposure during early growth

Principal Component analysis (PCA) was employed to investigate the effects of cold temperatures on membrane phospholipid metabolism in both leaf and root of two silage corn genotypes (Figure 3.1 and 3.2). For the leaf, the first two components explained 71.42% of the total variance in the data, where principal component 1 (F1) explained 43.66% and principal component 2 (F2) explained 27.76% of the total variance, respectively (Figure 3.1A). The 9 different phospholipids separated the temperature treatments and two silage corn genotypes into four quadrants (Q) of the PCA. The PCA observation plot showed clear segregation of five temperature regimes, where 25 °C and 20 °C were grouped in the left region of F1 (Q1 and Q2), whereas 10 °C and 5 °C were observed in the right region of F1 (Q3 and Q4), while 15 °C was located in the middle region of these groups (Figure 3.1A). In addition, Yukon-R and A4177G3-RIB were observed on different sides of F2. Specifically, Yukon-R was shown in the upper region (Q1), while A4177G3-RIB appeared in the lower region (Q4) (Figure 3.1A). The PCA Biplot revealed the difference and similarities of 9 leaf phospholipids groups between five temperatures and across both genotypes (Figure 3.1B). For example, PS, PI, LPE, PG and PC were clustered in Q1 and Q2, and were positively associated with higher growth temperatures (25° C and 20 °C). In contrast, PE, PA, LPC and LPG were grouped in the opposite regions (Q3 and Q4). Interestingly, the biplot suggests that PA, PE and LPC had strong negative correlations with PC and PG (Figure 3.1B).

Figure 3.2 has shown the association between different temperature treatments and root membrane phospholipids of two silage corn genotypes. The PCA component F1 explained 41.37% and F2 explained 24.06% of total variance in the date, respectively. These first two components accounted

for 65.43% of the total variability in the data (Figure 3.2A). The segregations between different growth temperatures and genotypes were also found in the observation plot (Figure 3.2A). Normal growth temperatures (25 °C and 20 °C) were grouped together in the same quadrant (Q2), while the lower growth temperatures (10 °C and 5 °C) were clustered together in the opposite quadrant (Q3), 15 °C was grouped in the intermediate region (Figure 3.2A). Both silage corn genotypes (Yukon-R and A1477G3-RIB) clustered in Q4 and Q1, respectively, with the following phospholipids: PA, PC, PE, PG, PI, PS, LPA, LPC and LPE (Figure 3.2A&B). The Biplot observed following PCA demonstrated the relationships of root phospholipids affected by different treatments (Figure 3.2B). PC and PG were observed in Q2, which were positively associated with non-cold temperatures. However, PA, PE, and LPC were shown in the same quadrant as 10 °C and 5 °C, suggesting an association between cold temperatures and the expressed levels of these phospholipids (Figure 3.2B). Besides, PI was negatively correlated to LPE, LPG and PS (Figure 3.2B).

A



B

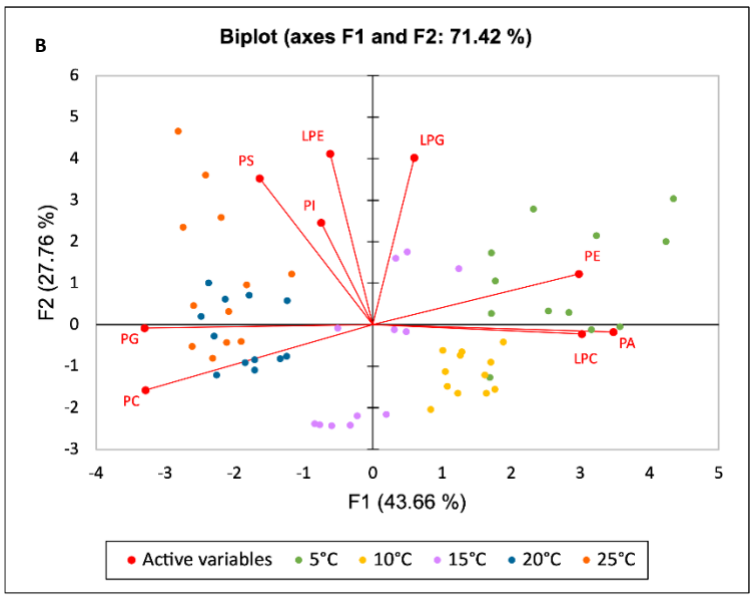


Figure 3.1 Principal component analysis (PCA) showing the separation of different silage corn leaf membrane phospholipids in response to cold temperatures. Observation plot showing the separation of the silage corn genotypes in response to different growth temperature exposure during early growth (**A**). Biplot showing the 9 important phospholipids and associated groupings of the silage corn genotypes following exposure to different cold temperatures during early growth (**B**). The phospholipids detected were: phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylglycerol inositol (PI), phosphatidylglycerol serine (PS), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) and lysophosphatidylglycerol (LPG).

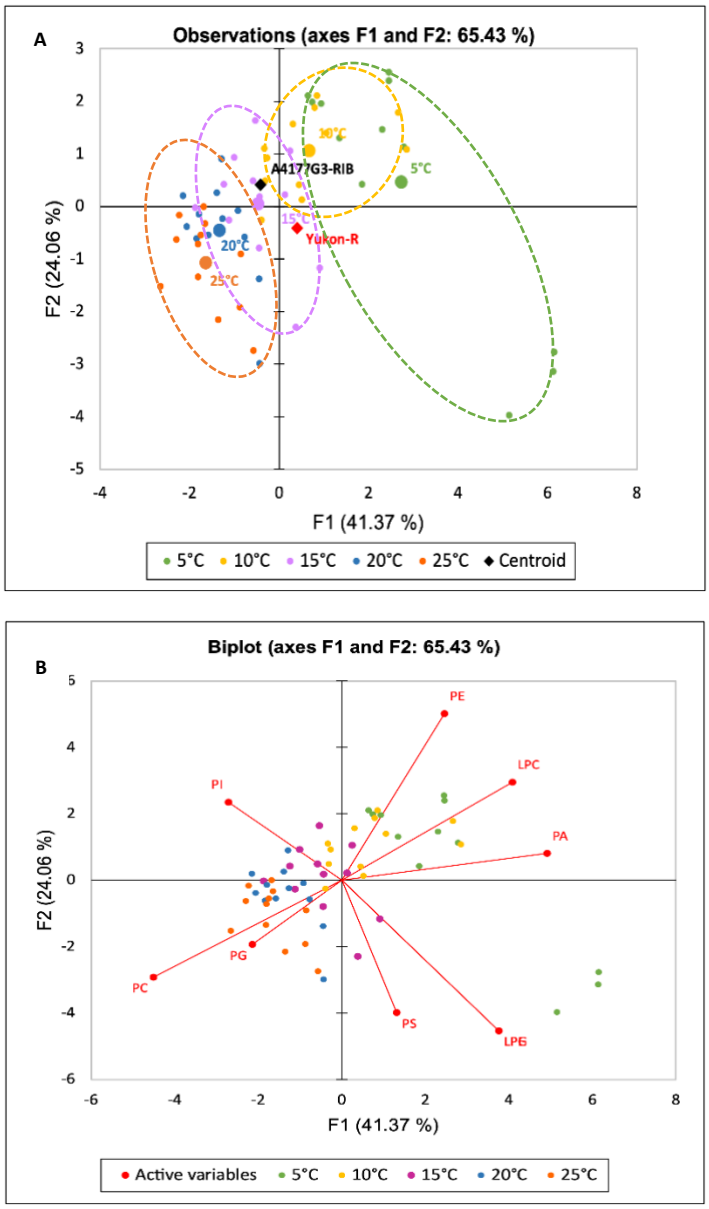


Figure 3.2 Principal component analysis (PCA) showing the separation of different silage corn root membrane phospholipids in response to cold temperatures. Observation plot showing the separation of the silage corn genotypes in response to different growth temperature exposure during early growth (**A**). Biplot showing the 9 important phospholipids and associated groupings of the silage corn genotypes following exposure to different cold temperatures during early growth (**B**). The phospholipids detected were: phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylglycerol inositol (PI), phosphatidylglycerol serine (PS), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) and lysophosphatidylglycerol (LPG).

3.3.3. Phospholipids remodelling in silage corn membrane in response to cold stress

Analysis of variance following PCA depicted significant phospholipid remodelling in silage corn seedling leaf and root membranes following exposure to cold stress (low growth temperatures) during early growth. Firstly, significant decreases (p<0.001) in PC were recorded in both leaf and root under different temperatures (Figure 3.4). In silage corn leaf, a continuous reduction of PC was found, and the highest PC content (70.07±0.57 nmol%) was noted when seedlings were grown at 25 °C, whereas the lowest level (54.14±1.02 nmol%) was observed at 5 °C (Figure 3.4A). Similarly,, the highest PC content was observed in silage corn roots exposed to a growth temperature of 25 °C (67.75±0.42 nmol%), while the lowest value was seen in plants grown at 5 °C (55.90±0.82 nmol%) (Figure 3.4B).

Growth temperature and the interaction of temperature and genotype expressed significant effects (p<0.001 and p=0.003), respectively, on the PE content in the leaf membrane of silage corn. Temp × Geno interaction resulted in significantly higher PE content (23.39±1.62 nmol%) in A4177G3-RIB at 5 °C, whereas the lowest PE (15.31±0.77 nmol%) was recorded at 15 °C in the same genotype (Figure 3.5A). In addition, the PE contents were significantly higher in A4177G3-RIB than in Yukon-R at 10 °C and 5 °C (Figure 3.5A). Growth temperature had significant effects (p<0.001) on root PE content. The highest PE contents (20.83±1.03 nmol%), were recorded at 5 °C and the lowest (15.75±0.37 nmol%) was noted at 25°C (Figure 3.5B). A trend of increasing PE contents was found to occur concomitantly with decreasing growth temperature during early seedling growth (Figure 3.5B).

Likewise, growth temperature displayed significant effects (p<0.001) on leaf and root PG contents (Figure 3.6 A, B). Interestingly, significant differences (p=0.036 and p<0.001, respectively) in PG contents were observed in both silage corn genotypes (Figure 3.6 C, D). However, there were no interactions between growth temperature and silage corn genotypes with respect to the level of PG in silage corn leaf and root membrane. Significant higher PG in silage corn leaf (10.49±0.25 nmol%) and root (5.52±0.35 nmol%) were recorded at 25 °C, whereas the lowest quantities were found at 5 °C (Figure 3.6 A, B). In comparison, the two silage corn genotypes, Yukon-R expressed significantly higher PG levels in both leaf (8.88±0.32 nmol%) and root (5.09±0.20 nmol%) membranes than A4177G3-RIB (Figure 3.6 C, D).

As shown in Figure 3.7, growth temperature had significant effects (p<0.001) on PA levels in leaf and root. Significantly higher PA contents were recorded at 5 °C treatment in leaf (11.53±0.37 nmol%) and root (11.92±0.83 nmol%) membranes (Figure 3.7 A, B). Likewise, silage corn genotypes had significant effects on PA content in leaf and root (p<0.001 and p=0.017, respectively). Compared to genotype A4177G3-RIB, Yukon-R showed higher PA levels in leaf (8.00±0.65 nmol%) and root (7.89±0.74 nmol%) (Figure 3.7 C, D) following exposure to low growth temperature during early growth.

Growth temperature had significant (p<0.001) effects on LPC expression levels in both seedling leaf and root membranes (Figure 3.3). Significantly higher LPC contents (1.73±0.13 nmol%) were observed at 5 °C, whereas the lowest (0.46±0.10 nmol%) was recorded at 20 °C, which was statistically at par with plants grown at 25 °C (Figure 3.3A). Similarly, the root LPC contents were increased under cold stress conditions (10 °C and 5 °C) and the highest values (1.38±0.05 nmol%)

were recorded at 5 °C, whereas the lowest (0.26±0.06 nmol%) were observed at 25 °C (Figure 3.3B).

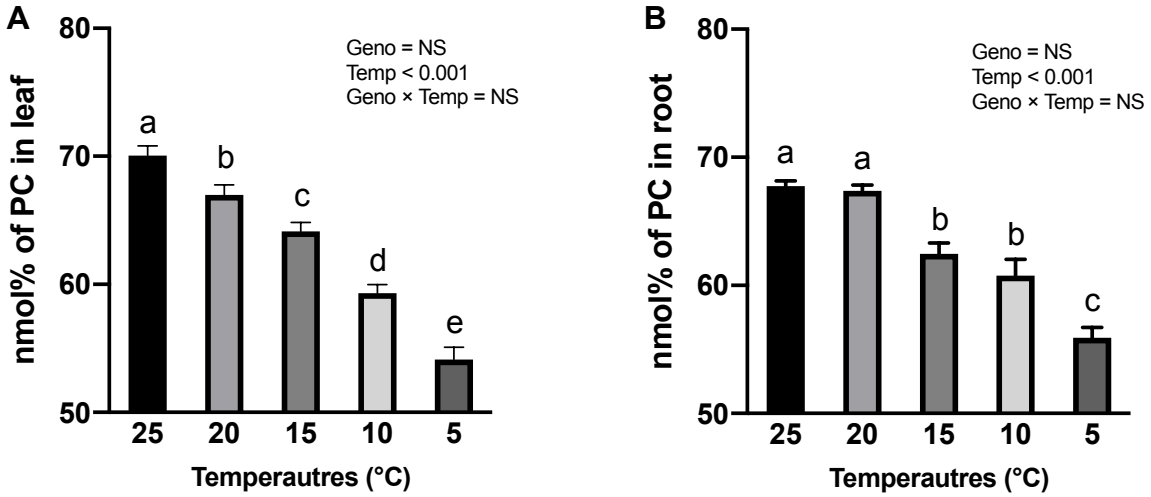


Figure 3.3 Effects of growth temperature on PC contents in silage corn leaf (**A**), and root (**B**). Each vertical bar represents the average of replicates ± SE (n=12). Different lowercase letters indicate significant differences among treatments at $p \leq 0.05$ according to Fisher's Least Significant test. Geno: genotype; Temp: temperature; PC: phosphatidylcholine.

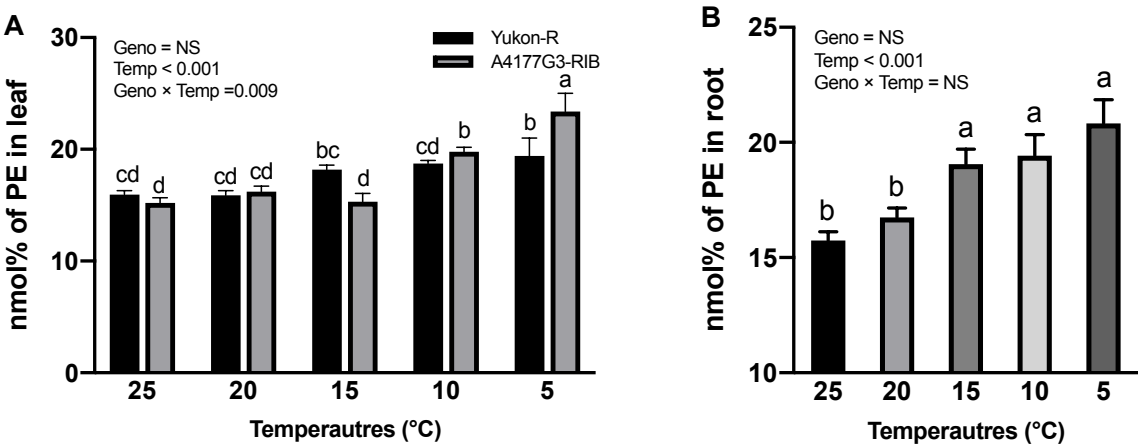


Figure 3.4 Interactive effects of growth temperature and silage corn genotypes on PE content in leaf membrane (**A**), effects of growth temperature on PE contents in silage corn root **(B)**. Each vertical bar represents the average of replicates ± SE (n=12 for Temp, n=6 for Geno × Temp). Different lowercase letters indicate significant differences among treatments at $p \leq 0.05$ according to Fisher's Least Significant test. Geno: genotype; Temp: temperature; PE: phosphatidylethanolamine.

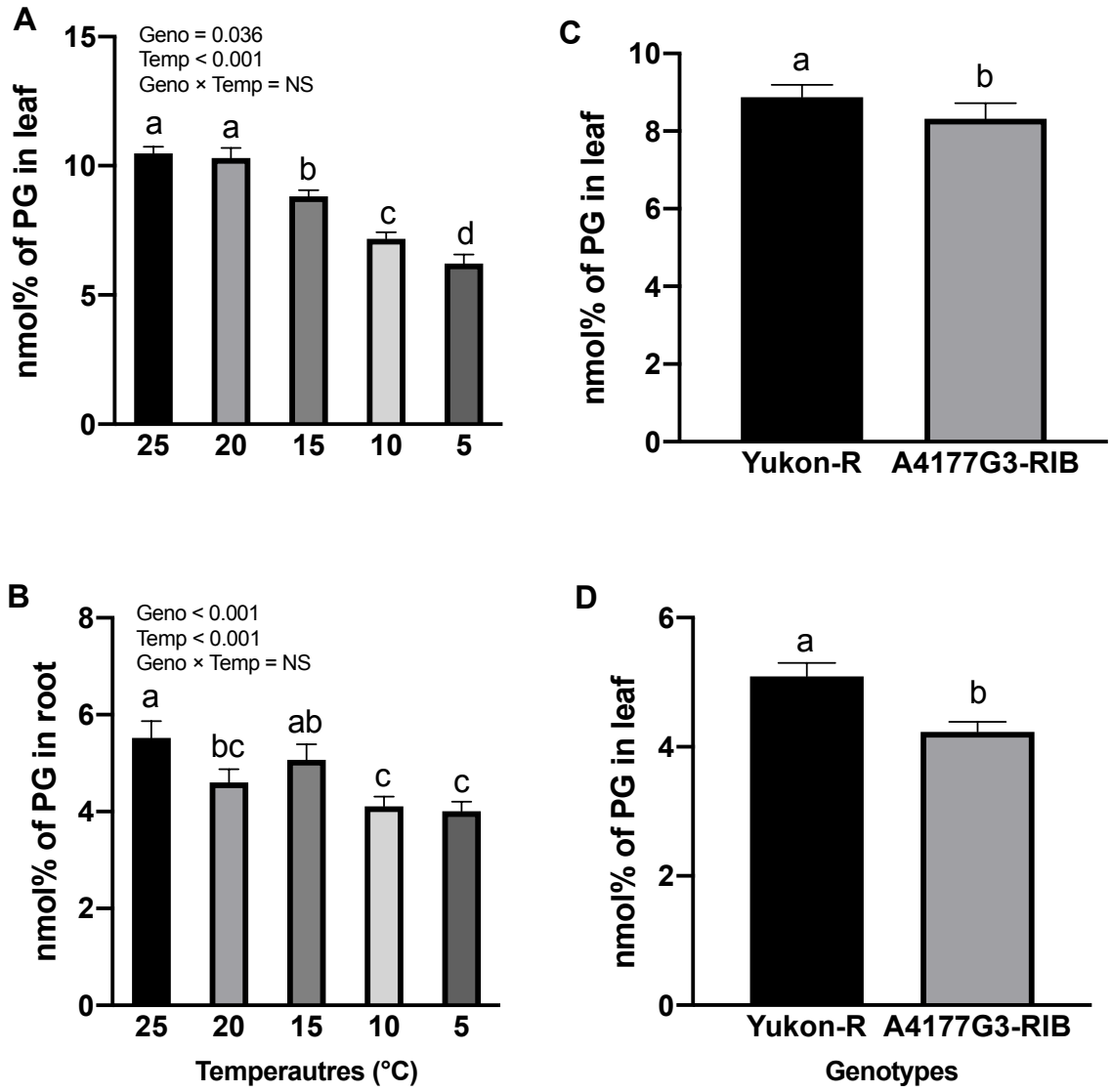


Figure 3.5 Effects of growth temperature on PG contents in silage corn leaf membrane (**A**), and root (**B**); the response of silage corn genotypes to PG contents in leaf (**C**), and root (**D**). Each vertical bar represents the average of replicates ± SE (n=30 for Geno, n=12 for Temp) . Different lowercase letters indicate significant differences among treatments at $p \leq 0.05$ according to Fisher's Least Significant test. Geno: genotype; Temp: temperature; PG: phosphatidylglycerol.

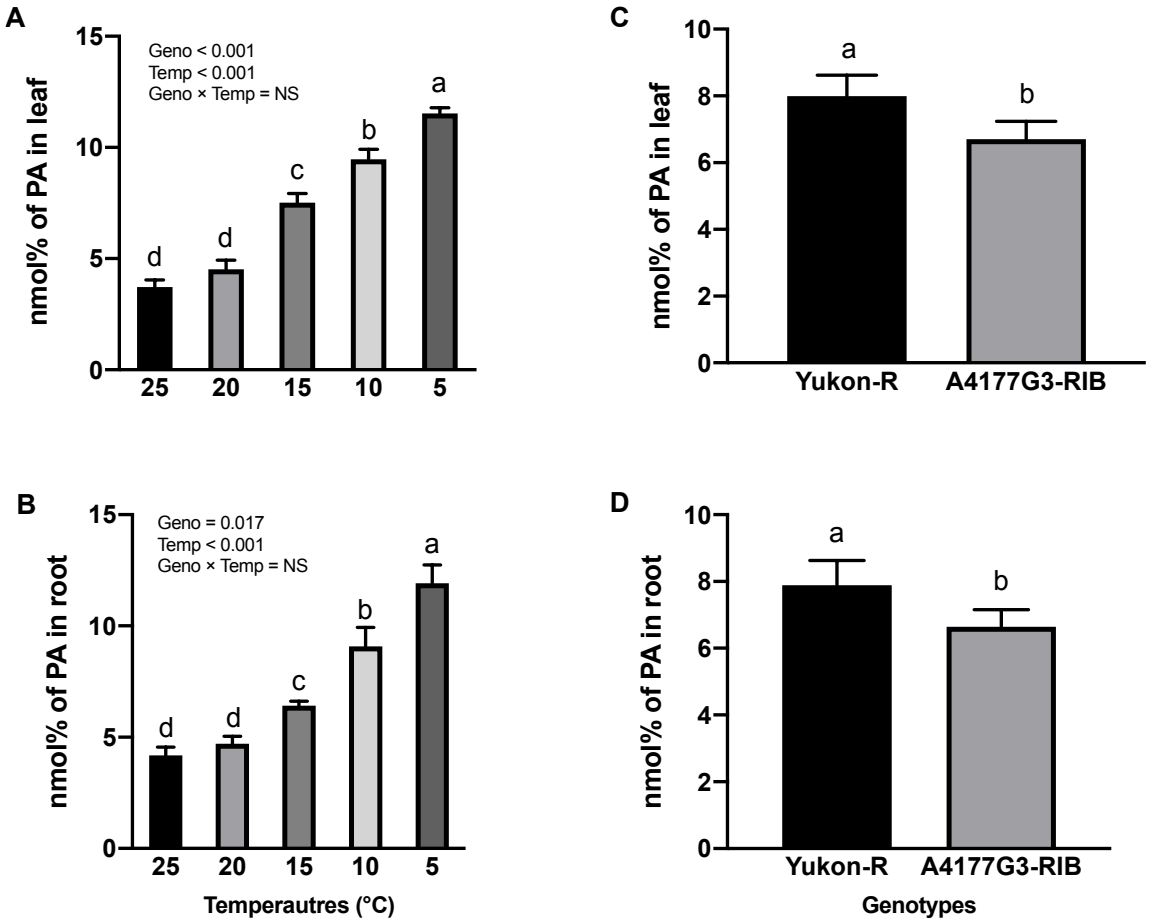


Figure 3.6 Effects of growth temperature on PA contents in silage corn leaf (**A**), and root (**B**); the response of silage corn genotypes to PG contents in leaf (**C**), and root membranes (**D**). Each vertical bar represents the average of replicates ± SE (n=30 for Geno, n=12 for Temp). Different lowercase letters indicate significant differences among treatments at $p \leq 0.05$ according to Fisher's Least Significant test. Geno: genotype; Temp: temperature; PA: phosphatidic acid.

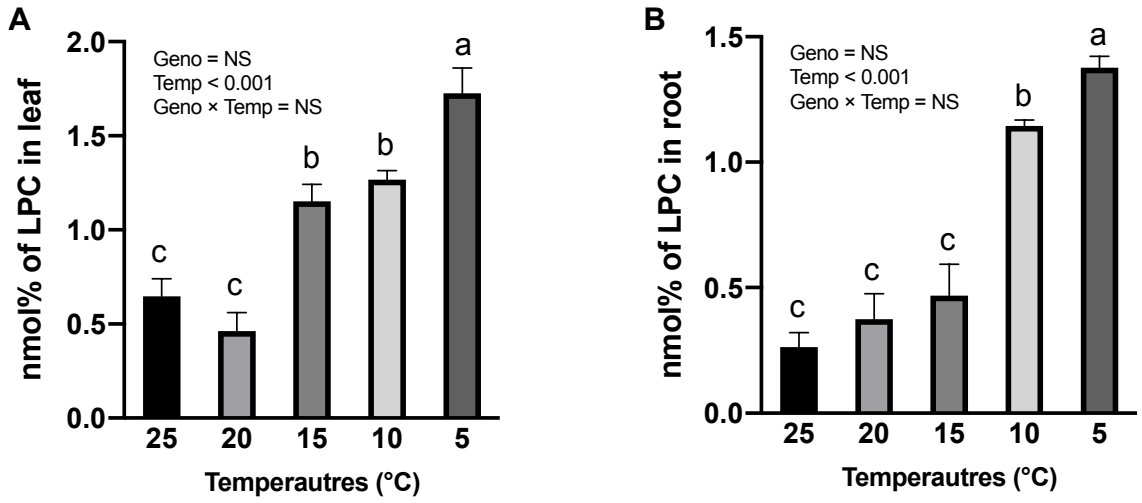


Figure 3.7 Effects of growth temperature on LPC contents in silage corn leaf (**A**), and root (**B**). Each vertical bar represents the average of replicates ± SE (n=12). Different lowercase letters indicate significant differences among treatments at $p \leq 0.05$ according to Fisher's Least Significant test. Geno: genotype; Temp: temperature; LPC: lysophosphatidylcholine.

3.3.4. Expression of PA synthesis genes in silage corn genotypes exposed to low temperatures during early growth

Compared to 25 °C, the expression levels of *ZmPLDα1* and *ZmPLDα2* in Yukon-R and A4177G3-RIB were up-regulated under cold stress (Figure 3.8 A, B). The *ZmPLDα1, ZmPLDα2* mRNA reached peak levels (3.23 and 5.07-fold, respectively) in Yukon-R at 15 °C, whereas the highest levels (2.36 and 4.38-fold, respectively) in A4177G3-RIB were observed at 10 °C (Figure 3.8 A, B). The expression patterns of *ZmPLDδ1* and *ZmPLDδ2* in Yukon-R and A4177G3-RIB reached the highest expression levels at 15 °C. However, the fold changes of *ZmPLDδ2* (9.52 in Yukon-R, 5.74 in A4177G3-RIB) when plants were exposed to a higher growth temperature of 25°C were much elevated than the levels of *ZmPLDδ1* (3.73 in Yukon-R, 2.57 in A4177G3-RIB) (Figure 3.8 C, D). The maximum transcript abundance of *ZmPLC1* was observed at 5 °C in Yukon-R (7.47-fold change), whereas the highest expression level in A4177G3-RIB was found at 20 °C (4.32-fold change) (Figure 3.8E). Two *DGK* genes from silage corn (*ZmDGK1* and *ZmDGK2*) were also stimulated by cold temperatures (Figure 3.8 F, G). The highest transcript levels of *ZmDGK1* in Yukon-R and A4177G3-RIB were found at 15 °C and 5 °C, respectively. The highest expression levels of *ZmDGK2* in both cultivars were noted at 15 °C (Figure 3.8 G). These results demonstrated that the alterations of *PLD*, *PLC* and *DGK* genes could be responsible for the PA accumulation under cold stress conditions during early growth in silage corn.

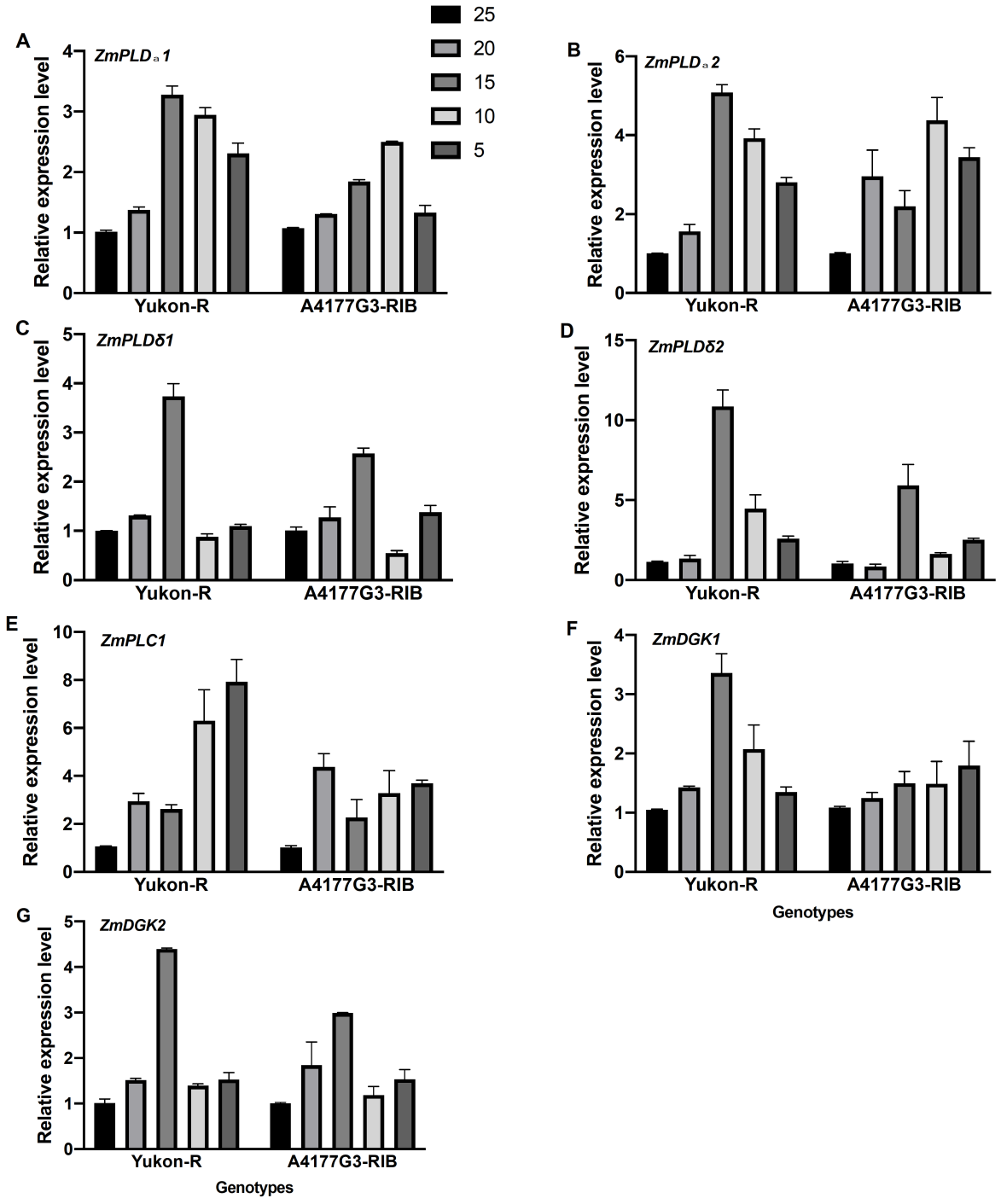
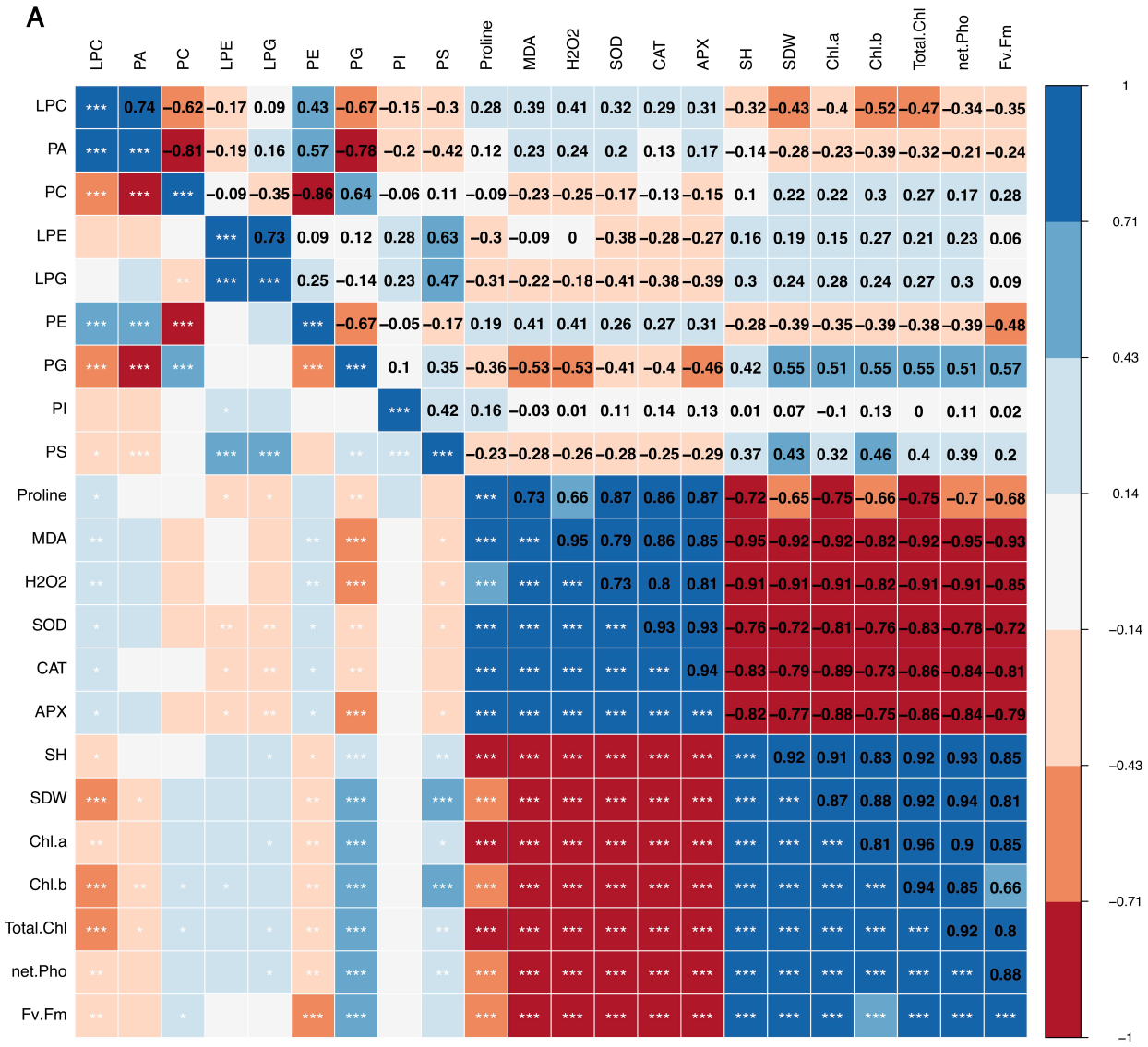


Figure 3.8 Relative transcript levels of PA synthesis genes in silage corn leaf. Expression of *ZmPLDα1* (**A**), *ZmPLDα2* (**B**), *ZmPLDδ1* (**C**), *ZmPLDδ2* (**D**), *ZmPLC1* (**E**), *ZmDGK1* (**F**), *ZmDGK2* (**G**) in two silage corn genotypes and different growth temperature. Each vertical bar represents the average of triplicates ± SE of the mean. PA: phosphatidic acid.

3.3.5. Correlations between phospholipids and plant morphological, physiological, and biochemical parameters following silage corn exposure to cold stress during early growth

Pearson's correlation analysis was performed to assess the relationships between different phospholipids and phenotypic indicators (morphological, physiological and biochemical) of silage corn seedling leaves and roots response to cold stress (Figure 3.9). Figure 3.9A showed that PC, PG, PS, LPE and LPG were positively correlated with morphological (SDW, SH) and physiological parameters (F_v/F_m, *Chl a, Chl b*, total chlorophyll, and photosynthetic rate) in silage corn leaf when exposed to cold stress during early growth. Furthermore, positive correlations were also found between three other phospholipid sub-classes (PA, PE and LPC) and biochemical parameters, including MDA, H_2O_2, proline CAT and APX (Figure 3.9A). PC, PG, LPE and LPG had positive correlations with root morphological parameters (RDW, RSA, RL and RV), whereas PA, PE, PI, PS and LPC were negatively correlated (Figure 3.9B).


A
LPC PA PC LPE LPG PE PG PI PS Proline MDA H2O2 SOD CAT APX SH SDW Chl.a Chl.b Total.Chl net.Pho Fv.Fm
LPC *** 0.74 −0.62 −0.17 0.09 0.43 −0.67 −0.15 −0.3 0.28 0.39 0.41 0.32 0.29 0.31 −0.32 −0.43 −0.4 −0.52 −0.47 −0.34 −0.35
PA *** *** −0.81 −0.19 0.16 0.57 −0.78 −0.2 −0.42 0.12 0.23 0.24 0.2 0.13 0.17 −0.14 −0.28 −0.23 −0.39 −0.32 −0.21 −0.24
PC *** *** *** −0.09 −0.35 −0.86 0.64 −0.06 0.11 −0.09 −0.23 −0.25 −0.17 −0.13 −0.15 0.1 0.22 0.22 0.3 0.27 0.17 0.28
LPE *** 0.73 0.09 0.12 0.28 0.63 −0.3 −0.09 0 −0.38 −0.28 −0.27 0.16 0.19 0.15 0.27 0.21 0.23 0.06
LPG ** *** *** 0.25 −0.14 0.23 0.47 −0.31 −0.22 −0.18 −0.41 −0.38 −0.39 0.3 0.24 0.28 0.24 0.27 0.3 0.09
PE *** *** *** *** −0.67 −0.05 −0.17 0.19 0.41 0.41 0.26 0.27 0.31 −0.28 −0.39 −0.35 −0.39 −0.38 −0.39 −0.48
PG *** *** *** *** *** 0.1 0.35 −0.36 −0.53 −0.53 −0.41 −0.4 −0.46 0.42 0.55 0.51 0.55 0.55 0.51 0.57
PI * *** 0.42 0.16 −0.03 0.01 0.11 0.14 0.13 0.01 0.07 −0.1 0.13 0 0.11 0.02
PS * *** *** *** ** *** *** −0.23 −0.28 −0.26 −0.28 −0.25 −0.29 0.37 0.43 0.32 0.46 0.4 0.39 0.2
Proline * * * ** *** 0.73 0.66 0.87 0.86 0.87 −0.72 −0.65 −0.75 −0.66 −0.75 −0.7 −0.68
MDA ** ** *** * *** *** 0.95 0.79 0.86 0.85 −0.95 −0.92 −0.92 −0.82 −0.92 −0.95 −0.93
H2O2 ** ** *** * *** *** *** 0.73 0.8 0.81 −0.91 −0.91 −0.91 −0.82 −0.91 −0.91 −0.85
SOD * ** ** * ** *** *** *** *** 0.93 0.93 −0.76 −0.72 −0.81 −0.76 −0.83 −0.78 −0.72
CAT * * ** * ** *** *** *** *** *** 0.94 −0.83 −0.79 −0.89 −0.73 −0.86 −0.84 −0.81
APX * ** * ** * *** * *** *** *** *** *** *** −0.82 −0.77 −0.88 −0.75 −0.86 −0.84 −0.79
SH * * * * *** ** *** *** *** *** *** *** *** 0.92 0.91 0.83 0.92 0.93 0.85
SDW *** * ** *** *** *** *** *** *** *** *** *** *** *** 0.87 0.88 0.92 0.94 0.81
Chl.a ** * ** *** *** *** *** *** *** *** *** *** *** *** *** 0.81 0.96 0.9 0.85
Chl.b *** ** * * ** *** *** *** *** *** *** *** *** *** *** *** *** 0.94 0.85 0.66
Total.Chl *** * * * ** *** ** *** *** *** *** *** *** *** *** *** *** *** *** 0.92 0.8
net.Pho ** * ** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** 0.88
Fv.Fm ** * *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
1
0.71
0.43
0.14
−0.14
−0.43
−0.71
−1

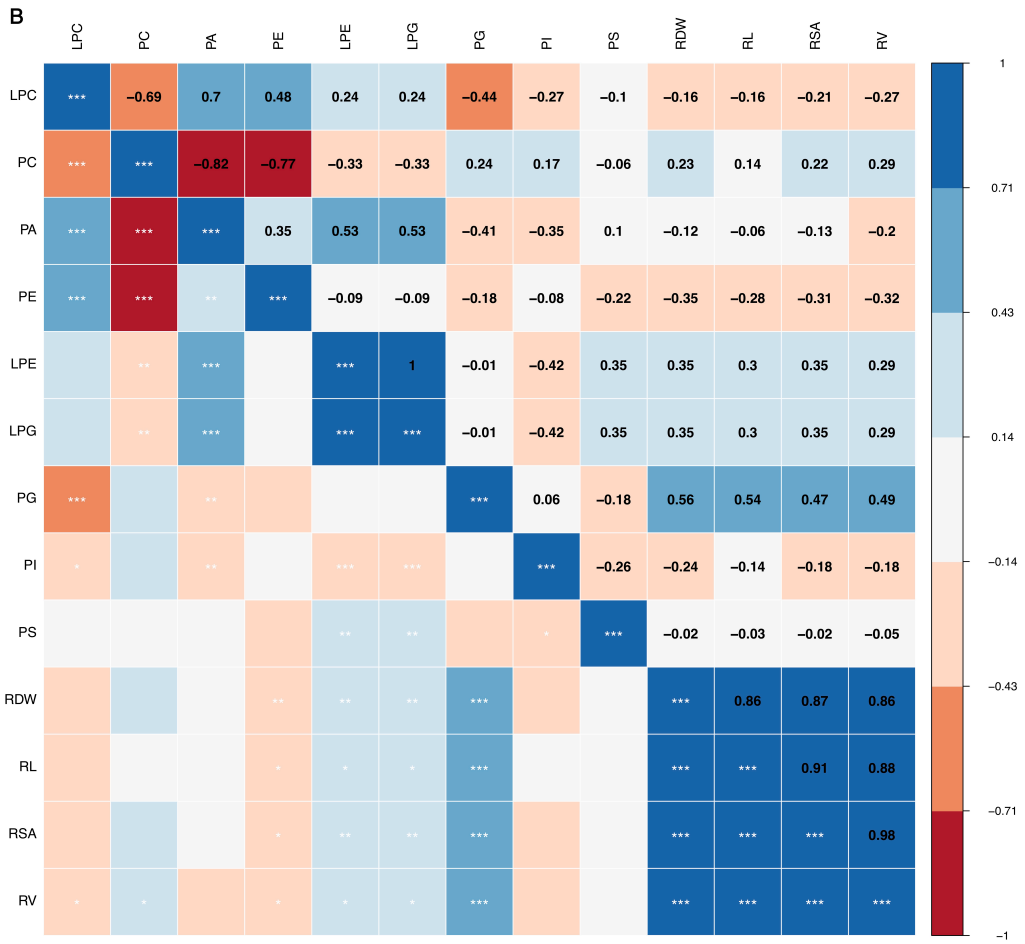


Figure 3.9 The Pearson's correlation matrixes showing the association between different morphological, physiological or biochemical parameters and the remodeled membrane phospholipids in silage corn genotypes exposed to cold stress during early growth. Correlation matrix between morphological, physiological or biochemical parameters and phospholipids from leaf membrane of silage corn (**A**); Correlation matrix between morphological, physiological or biochemical parameters and phospholipids from root membrane of silage corn (**B**).
*, ** and *** indicate the $p \leq 0.05$, 0.01 and 0.001 respectively. APX: ascorbate peroxidase; CAT: catalase; SOD: superoxide dismutase; Chl *a*: chlorophyll *a*; Chl *b*: chlorophyll *b*; H_2O_2: hydrogen peroxide; MDA: malondialdehyde; RDW: root dry weight; RL: root length; RSA: root surface area; RV: root volume; SDW: shoot dry weight; SH: seedling height; total Chl: total chlorophyll. PA: phosphatidic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, PI: phosphatidylglycerol inositol, PS: phosphatidylglycerol serine, LPC: lysophosphatidylcholine, LPE: lysophosphatidylethanolamine, LPG: lysophosphatidylglycerol.

3.4. Discussion

Silage corn is considered a cold-sensitive crop species, and there is limited understanding of the adaptation mechanisms used in this crop during the successful response to circumvent cold temperature stress. Lipidomic analysis allows the quantification and identification of plant membrane lipids and assesses their functions in response to stress conditions (Pham et al. 2019). However, most of the lipidomics studies were conducted in the grain corn, and only focused on one tissue (above or under-ground). Thus, very little is known about how the silage corn leaf and root membrane lipid metabolism changes under cold and non-cold temperature stress. In the present study, the membrane phospholipidome of two silage corn genotypes exposed to cold stress during early growth was assessed to better understand the adaptation mechanism used by silage corn to successfully circumnavigate or acclimate to cold temperatures.

3.4.1. Cold-induced phospholipidome remodeling in silage corn cellular membrane in response to low temperature at early seedling growth

Cold temperature stress generally induces membrane remodelling to maintain cellular membrane integrity. Phospholipids represent a major class of lipids in plant tissues, which are essential components of plasma membranes and signaling molecules in plants (Kehelpannala et al. 2021; Nakamura 2017). Besides, phospholipids are essential structural lipids that build up cellular membranes, and silage corn phospholipids accounted for the highest subclass of total leaf and root lipid classes, irrespective of temperatures and genotypes (Table 3.1 and Table 3.2).

As shown in Figure 3.1 and 3.2, the output from the PCA observation plot showed segregation of five growth temperatures into three distinct groups based on phospholipids sub-classes present in

silage corn leaf and root membranes. Moreover, the phospholipid composition changes observed in silage corn seedlings were correlated to growth temperatures rather than to their genetic traits. The correlations between these phospholipids were shown in different quadrants of the PCA biplots (Figure 3.1B and 3.2B). Such variations among phospholipid sub-classes also showed great potential to screen cold-tolerant varieties/genotypes under cool climate conditions (Nadeem et al. 2020). Overall, PCA of leaf and root indicates that remodelling of some phospholipids, including PC, PE and PA, were highly correlated to changes in growth temperature during early growth, suggesting that some phospholipids may play critical roles in plant cold acclimation (Cowan 2006; Yadav 2010).

3.4.2. Role of LPC, PA PC, PE, and PG in cold stress adaption of silage corn

PC and PE are the two most abundant phospholipid classes of extraplastidic membranes in plants (Bagatolli and Stock 2021; Jouhet et al. 2007; Michaud et al. 2017). We found that PC content was significantly decreased in silage corn leaf and root exposed to cold temperature stress during early growth. Compared to 25 °C, the PC contents of the leaf and root were reduced by 22.74% and 17.49%, respectively, at 5 °C (Figure 3.4). On the contrary, cold stress (5 °C) increased the amounts of PE in leaf and root by 18.49% and 24.40%, respectively, in comparison with cultivation at 25 °C (Figure 3.5). It is believed that the reduction of PC content during cold stress might result in membrane damage (Li et al. 2015). In fact, the reason for reduced PC (degradation) may be related to activities of different phospholipases that can hydrolyze the ester bonds linking the moieties at sn-3, sn-1 or sn-2 position of the glycerol backbone in PC (Meijer and Munnik 2003). The lipid bilayer is one of the fundamental structures of biological membranes, and environmental changes such as temperature usually cause a phase transition (Matsuki et al. 2013). PC exhibits a

cylindrical shape and lamellar phase, whereas PE can pack into hexagonal H_{II} structure due to its cone shape (Reszczyńska and Hanaka 2020). It is reported that a higher PC/PE ratio was highly associated with plasma membrane integrity (Welti et al. 2002). Wu et al. (2020) indicated that a higher ratio of PC/PE was responsible for protecting plant cells against stress damage. In our studies, the levels of PE in A4177G3-RIB were significantly higher than Yukon-R under severe cold conditions (10 °C and 5 °C). The previous results of MDA contents also confirmed that Yukon-R performed better in maintaining leaf membrane integrity under cold conditions (Figure 2.5B).

Most phospholipid classes (PC, PE, PA, PS, PI, etc.) are usually synthesized and predominate in extraplastidic membranes (Higashi and Saito 2019; Nakamura 2017). However, PG is the only phospholipid class synthesized in the plastids, which is critical for the function of photosystem II (PS-II) during photosynthesis (Sun et al. 2011; Wada and Murata 2007). Previous studies also demonstrated that the PG content varied between different crop species under stress conditions (Marla et al. 2017; Wu et al. 2020; Yu et al. 2019; Zheng et al. 2016). Our results indicated that the PG content was decreased by 40.70% at 5 °C in silage corn leaf compared to when the plants were grown at 25 °C. Yukon-R maintained significantly higher PG abundance in the leaf than A4177G3-RIB, which is consistent with our previous findings that A4177G3-RIB seedlings were more sensitive to PS II photoinhibition under cold stress (Figure 3.6C and 2.3C). Higher PG levels may repair cold-induced chloroplast damage and stabilize photosynthetic processes (Guo et al. 2019). In addition, a similar trend was found for the root PG levels in response to cold temperatures, and these were significantly different between the two cultivars. This could be explained by PG being a bilayer-forming lipid like PC, suggesting PG also plays a critical role in maintaining plasma membrane integrity (Pan et al. 2012; Salama et al. 2007). PG is classified as an anionic

membrane phospholipid. In plants, PG synthesis also requires the lipid procurer PA. PA is converted to cytidine diphosphate (CDP)-DAG by CDP-DAG synthase, which is then converted to PG phosphate (PGP) by PGP synthase. In the last step, the phosphate group is released by PGP to produce PG (Wada and Murata 2007).

PA acts as a precursor in the biosynthesis of phospholipids, galactolipids and triacylglycerols, and serves as second messengers in plant stress response (Margutti et al. 2017; Pokotylo et al. 2018). The cellular PA levels experience dynamic changes during different growth stages and under stress conditions (Hou et al. 2016; Wang et al. 2006). Accumulation of PA was found in numerous studies to occur concomitantly with plants exposure to cold stress or low growth temperatures (Gu et al. 2017; Gu et al. 2018; Zhang et al. 2020; Zhao et al. 2021). Results from our study showed that the PA contents were significantly accumulated in response to cold temperatures (15 °C, 10 °C and 5 °C) during early growth in silage corn. In fact, PA levels of silage corn leaf and root membranes were increased by 67.7% and 64.8% at 5°C, respectively, compared with growth at 25 °C. Previous studies reveal that the increase of PA could help corn seedlings alleviate cold injury (Zhao et al. 2021). Compare with A4177G3-RIB, the PA content in the leaf and root of Yukon-R increased by 1.20 and 1.18-fold, respectively. Nadeem et al. (2019b) demonstrated that higher PA levels in silage corn genotypes enhanced agronomic performance in cool climates. Moreover, PA plays an important role in mediating plant growth, and cellular and other abiotic stress response. It is possible that these PA-protein interactions could positively regulate cold tolerance. PA is involved in ROS and MAPK response pathways, which are considered potential targets related to cold tolerance (Park et al. 2004; Zhang et al. 2003). For instance, The PLDα1-derived PA could bind two NADPH oxidases (RbohD and RbohF) to produce ROS in response to ABA-dependent stomatal closure (Zhang et al. 2009). PLDδ also plays a potential role in ROS

perception and response to plant external stresses (Song et al. 2020). Yu et al. (2010) used a protein-lipid binding assay to discover that PA can interact with MPK6 in response to salt stress, which is also a cold-responsive kinase (Li et al. 2017).

Lysophospholipids are synthesized by hydrolysis of phospholipids by phospholipase A (PLA), which plays a vital role in mediating plant development and stress response (Adigun et al. 2021; Cowan 2006). In the present study, LPC contents from silage corn leaf and root membranes were increased 2.7 and 5.3-fold, respectively, when seedlings were exposed to a growth temperature of 5 °C compared to a temperature of 25 °C. Welti et al. (2002) also found that LPC increased dramatically in *Arabidopsis* under cold stress. The increased LPC contents indicated that low temperatures could strengthen lipolytic activity to produce more lipid intermediates in response to cold stress (Li et al. 2014; Wang 2004; Zheng et al. 2016).

3.4.3. Accumulation of PA is associated with *PLD*, *PLC* and *DGK* expression

Results from the assessment of silage corn leaf and root membrane lipidome demonstrated that the PA levels were markedly increased during cold stress conditions. The PA accumulation under stress conditions is attributed to phospholipases and lipid kinase activation (Ruelland et al. 2015). In this study, the changes in the expression of PA synthesis-related genes from two pathways to determine how transcriptional regulation would be affected by cold temperature stress were evaluated (Figure 3.10). The corn genome is predicted to encode 13 *PLDs*, 11 *PLCs* and 7 *DGKs* (Chen et al. 2017; Gu et al. 2018; Zhu et al. 2021). Based on previous studies, seven genes from the PLD family (*ZmPLDα1, ZmPLDα2, ZmPLDδ1* and *ZmPLDδ2*), PLC family (*ZmPLC1*), and DGK family (*ZmDGK1*, *ZmDGK2*) were selected (Chen et al. 2017; Gu et al. 2018; Wang et al. 2008; Zhai et al. 2005). Our results showed that *PLD*, *PLC* and *DGK* genes were all up regulated

when silage corn was exposed to cold temperatures (15 °C, 10 °C and 5 °C) during early growth stages; these findings are consistent with previous transcriptomic analyses (Gu et al. 2017; Zhang et al. 2020; Zhao et al. 2021). Most of these genes reached the highest expression levels at 15 °C (Figure 3.8). Genetically modified plants have been used to address the role of PLDδ-derived PA in response to cold stress (Li et al. 2004; Yang et al. 2021), and higher expression of *ZmPLDδ1* and *ZmPLDδ2* in our study might have played a positive role in silage corn cold tolerance (Figure 3.8 C, D). However, PLDα1 is detrimental to cell membranes that preferentially hydrolyse PC to produce PA (Barrero-Sicilia et al. 2017; Kargiotidou et al. 2010; Welti et al. 2002). Our results showed that higher expression levels of *ZmPLDα1* and *2* under cold stress, might be responsible for the PC reduction in silage corn (Figure 3.8 A, B).

Although the precise functions of individual PLCs and DGKs in plants under cold stress tolerance remain undetermined due to a lack of published research. A few studies have reported that the PLC-DGK pathway and its derived PA played indispensable roles in regulating early ROS-mediated signaling pathways, which may be involved in cold response (Cacas et al. 2017). Our results showed that the expression of *ZmPLC1*, *ZmDGK1* and *ZmDGK2* were all up regulated during cold conditions, implying their potential role in silage corn cold stress response (Figure 3.8 E-F). Moreover, the highest expression level of these genes in Yukon-R was higher than in A4177G3-RIB under cold temperatures, which is responsible for the higher PA content in Yukon-R under cold stress (Figure 3.10). Taken together, these results indicated the alterations in the expression of *PLD*, *PLC* and *DGK* genes could be helpful for alleviating cold stress in silage corn exposed to low temperature during early growth.

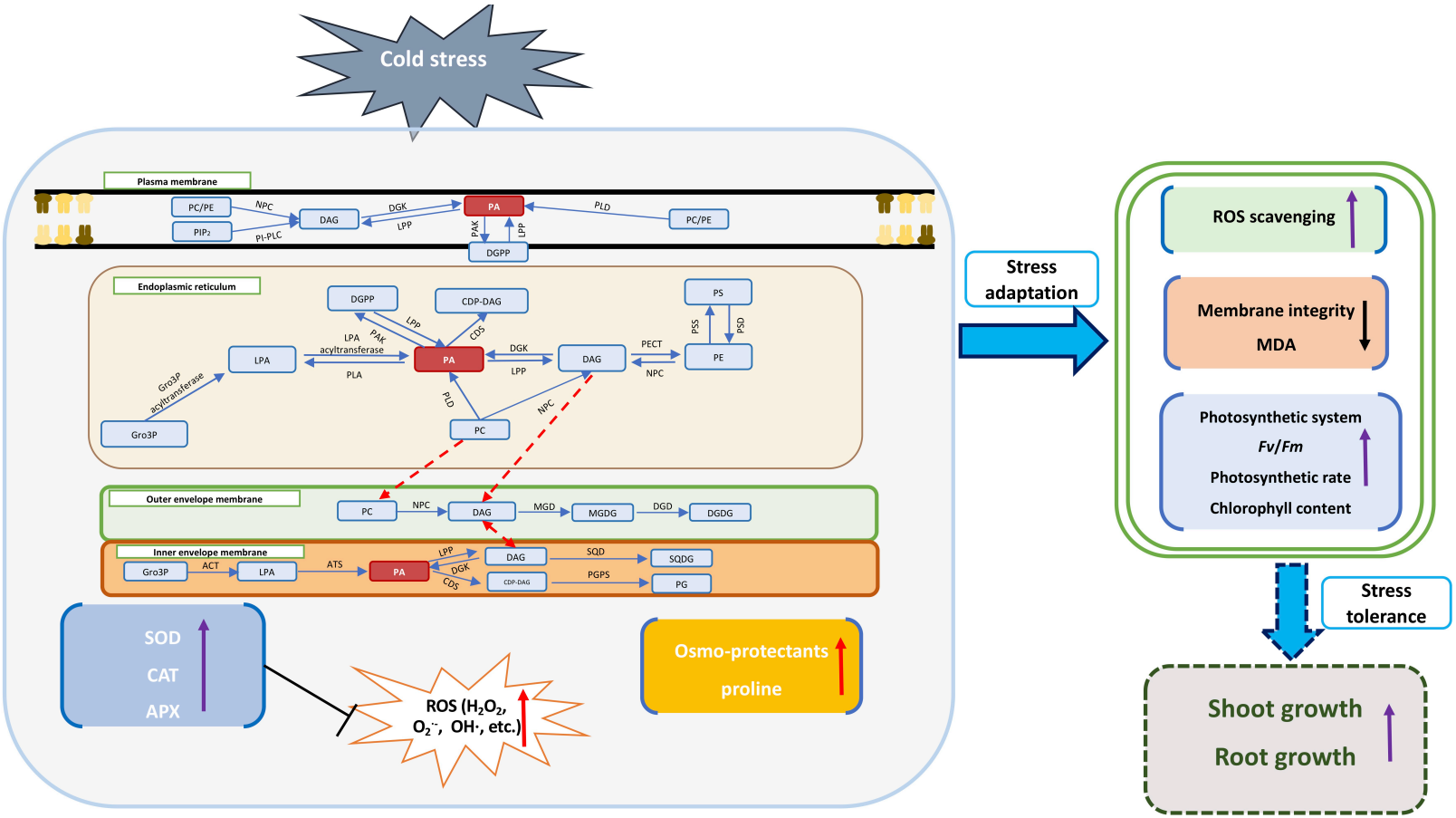

Cold stress
Plasma membrane
PC/PE
PIP2
NPC
PI-PLC
DAG
DGK
LPP
PA
PAK
LPP
PLD
PC/PE
DGPP
Endoplasmic reticulum
DGPP
CDP-DAG
LPP
PAK
CDS
LPA
acyltransferase
PLA
PA
DGK
LPP
DAG
PECT
NPC
PS
PSS
PSD
PE
Gro3P acyltransferase
LPA
Gro3P
PLD
PC
NPC
Outer envelope membrane
PC
NPC
DAG
MGD
MGDG
DGD
DGDG
Inner envelope membrane
Gro3P
ACT
LPA
ATS
PA
LPP
DGK
CDS
DAG
CDP-DAG
SQD
PGPS
SQDG
PG
SOD
CAT
APX
ROS (H2O2, O2·-, OH·, etc.)
Osmo-protectants
proline
Stress adaptation
ROS scavenging
Membrane integrity
MDA
Photosynthetic system
Fv/Fm
Photosynthetic rate
Chlorophyll content
Stress tolerance
Shoot growth
Root growth

Figure 3.10 Schematic representation of the phospholipidome metabolism and associated mechanisms in response to cold stress of silage corn at early growth stages. Cold stress affects silage corn seedling physiological, biochemical process, such as photosynthesis and redox hemostasis. The alteration of phospholipids is required to cope with cold stress. The cold stress adaption includes increasing ROS scavenging, polythetic system as well as decreasing the MDA content. These adaption strategies help silage corn shoot and root growth under cold temperatures. Lipid transports are indicated by a red semi-dashed line. Purple and black arrows indicate increase and decrease regulations, respectively. ACT: plastidial glycerpiol-3-phosphate acyltransferase; APX: ascorbate peroxidase; ATS: plastidial lysophosphatidic acid acyltransferase; CAT: catalase; CDP-DAG: cytidine diphosphate diacylglycerol; CDS: cytidine diphosphate diacylglycerol synthases; DAG: diacylglycerol; DGD: digalactosyldiacylglycerol synthetase; DGDG: digalactosyldiacylglycerol; DGK: diacylglycerol kinase; DGPP: diacylglycerol pyrophosphate; Gro3P: glycerpiol-3-phosphate; LPA: lysophosphatidic acid; MDA: malondialdehyde; MGD: monogalactosyl diacylglycerol synthase; MGDG: monogalactosyl diacylglycerol; NPC: non-specific phospholipase C; PA: phosphatidic acid; PAK: phosphatidic acid kinase; PAP: phosphatidic acid phosphatase; PC: phosphatidylcholine; PE: phosphatidylethanolamine; EPT: CDP-ethanolamine phosphotransferase; PG: phosphatidylglycerol; PGPS: phosphatidylglycerol phosphate synthase; PI: phosphatidylinositol; PI-PLC: phosphoinositide phospholipase C; PIP2: phosphatidylinositol 4,5-bisphosphate; PLA: phospholipase A; PLC: phospholipase C; PLD: phospholipase D; PS: phosphatidylserine; PSD: PS decarboxylase; PSS: phosphatidylserine synthase; SOD: superoxide dismutase; SQD: sulfoquinovosyl diacylglycerol synthase; SQDG: sulfoquinovosyl diacylglycerol.

3.5. Conclusion

Our results indicated the importance of phospholipid metabolism during the acclimation of silage corn to cold temperature stress. Phospholipids represented the most abundant lipid in silage corn leaf and root membranes at the early growth stage. Besides, the study established membrane phospholipid remodeling as a suitable biomarker-assisted approach for assessing cold stress tolerance in silage corn seedlings. LPC, PA and PE appeared to be positively correlated with cold temperatures (10 °C and 5 °C), whereas PC and PG were more correlated to non-cold conditions (25 °C and 20 °C). Cold stress remarkably affected the extraplastidic membrane integrity by reducing PC and enhancing the PE content or level in the cell membrane. Moreover, the reduction of PG during cold stress resulted in the disruption of the photosynthetic processes. Some lipid intermediates such as LPC and PA were accumulated under cold stress in both silage corn genotypes. PA is an essential phospholipid involved in plant stress response, and its accumulation is considered a successful strategy used in plant cold temperature adaption. Yukon-R showed higher PA contents in leaf and root membranes than A4177G3-RIB under cold stress conditions, which might be responsible for the superior cold tolerance observed in Yukon-R. Besides, transcription analysis indicated that higher PA synthesis in silage corn under cold temperature stress might be due to up-regulation of PA biosynthesis-related genes. This study lays the foundation for an improved understanding of the molecular regulators of silage corn mediating cold response and tolerance in the future. Further study is needed to identify the specific molecular species of different phospholipids, including PC, PE, PA, PG and LPC to better understand their roles in cold adaption, which would be helpful in developing cold resilient silage corn genotypes for the boreal ecosystem.

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Chapter 4
General Conclusions and Future Directions

4.1.Conclusion

Silage corn is considered a cold-sensitive crop, and therefore, morphological, physiological and biochemical processes associated with growth and yield are impacted under cold stress. There is limited understanding of the adaptation mechanisms of silage corn in boreal climate to circumvent cold stress. Additionally, very little is known about the silage corn leaf and root membrane lipid metabolism changes under cold stress and role of PA in cold stress tolerance. To answer these questions, experiments were conducted in a walk in growth chamber, and silage corn seedlings of Yukon-R and A4177G-RIB were grown at 25 °C up to V3 (three-leaf) stage and then subjected to five temperature regimes (25 °C, 20 °C, 15 °C, 10 °C, and 5 °C) for 5 days.

This thesis addressed two specific objectives as follows:

(**1**) To determine the effects of cold stress on the morphological, physiological, and biochemical responses of silage corn exposure to cold stress (low growth temperature) at the early growth stage.

Studies in chapter 2 demonstrated that the silage corn shoot and root growth were ultimately inhibited when exposed to cold temperatures. Besides, lower photosynthetic capacity and higher ROS accumulation were observed when the temperature decreased. In comparing two silage corn genotypes with varying cold stress tolerance; Yukon-R showed higher shoot biomass and lower hydrogen peroxide (H_2O_2) and (MDA) contents than A4177G3-RIB under cold stress.

In conclusion, chapter 2 showed that cold stress (15 °C, 10 °C and 5 °C) induces significant morphological, physiological, and biochemical changes in silage corn seedlings at the early growth

stage. Moreover, silage corn genotypes exhibited differential tolerance capacity in response to cold and non-cold stress. Genotype Yukon-R produced seedlings with higher shoot/root growth and lower H_2O_2 and MDA contents than A4177G3-RIB under cold stress. Over-accumulation of ROS and lipid peroxidation at the early growth stage contributed to significant reductions in the growth and root morphological traits of silage corn seedlings under cold stress conditions. The higher photosynthesis, proline content, and antioxidant enzymatic activities contributed to improved seedling biomass and cold tolerance in Yukon-R, suggesting that Yukon-R is potentially more suited for cultivations in boreal climate. This study provides useful information on potential silage corn genotypes with cold tolerant traits that may be suitable for cultivation in boreal climates.

(**2**) To investigate how the membrane lipidome metabolism mediates the cold stress tolerance of silage corn at the early growing stage.

Research in chapter 3 revealed that the phospholipid represented the most abundant lipid in silage corn leaf and root membrane at the early growth stage. Results indicated the importance of phospholipid metabolism during the acclimation of silage corn to cold temperature stress. Silage corn leaf and root tissues showed similar phospholipid remodeling trends. Lysophosphatidylcholine (LPC), phosphatidic acid (PA) and phosphatidylethanolamine (PE) appeared to be positively correlated to cold temperatures (10 °C and 5 °C), whereas phosphatidylcholine (PC) and phosphatidylglycerol (PG) were more correlated to non-cold conditions (25 °C and 20 °C). The remarkable decreases in PC and PG were responsible for disrupted cellular membrane integrity and photosynthetic processes. Besides, the study established membrane phospholipid remodeling as a suitable biomarker-assisted approach for assessing cold stress tolerance in silage corn seedlings. Some lipid intermediates such as LPC and PA were

accumulated under cold temperature stress in both silage corn genotypes. Yukon-R showed higher PA contents in leaf and root than A4177G3-RIB under cold stress, which might be responsible for the superior cold tolerance observed in Yukon-R. Transcription analysis also indicated that higher PA synthesis in silage corn under cold stress might be due to up regulation of PA biosynthesis-related genes.

In summary, studies of this dissertation uncovered the effects of cold stress on morphological, physiological and biochemical, as well as phospholipidome remodeling in silage corn at the early growth stage as part of a successful strategy to mitigate cold stress. Therefore, this research will further enhance our insights into understanding the cold tolerance mechanisms of silage corn and selecting superior genotypes for future field experiments to increase forage production in boreal climates.

4.2.Future directions

Cold stress reduces membrane fluidity, and fatty acid desaturation is thought to have effects on membrane integrity (Noblet et al. 2017; Reszczyńska and Hanaka 2020; Wada and Mizusawa 2009). Therefore, molecular species in different phospholipid sub-classes also play important roles in cold adaption. Future studies should identify the specific molecular species as well as changes in unsaturation in different phospholipids classes such as PC, PE, PA, PG and LPC under cold temperatures along with the genes (*ZmPLDs*, *ZmPLCs* and *ZmDGKs*) activated to better understand their roles in successful cold adaption.

Plant adaptation to cold stress is considered a dynamic process. The membrane lipid remodeling metabolism is controlled by transcriptional regulation (Barrero-Sicilia et al. 2017). Though

integrated lipidomic and transcriptomic analyses were conducted in grain corn, we still lack evidence of molecular regulation mediating silage corn response and acclimation to cold stress, particularly during early growth (Gu et al. 2017; Zhao et al. 2021). Such transcriptional analysis could help us to establish a lipid metabolic regulatory network that could effectively elucidate the cold response and tolerance mechanisms in silage corn.

The role of PA, as a second messenger, has been established by identifying PA-binding domains (PABD) within PA effectors in different plant cell processes (Testerink and Munnik 2005; Wang et al. 2006). However, target of PA during cold stress in plants is largely unknown. Therefore, further research is needed to find more PA-binding proteins to understand the mechanism of PA in cold stress response.

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