Adaptations to low temperatures in C_4 grasses

By

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Summary

Low temperatures significantly impact plant survival, particularly for species unable to acclimate to such conditions. Grasses, including those with C_3 photosynthesis, have shown remarkable resilience across broad temperature ranges, particularly in temperate and high-altitude regions with low mean annual temperatures. Although C_4 grasses typically originate from warmer climates, some can adapt to and thrive in colder environments. Given the importance of C_4 grasses like maize, sugarcane, and sorghum as food sources, understanding how C_4 grasses respond and adapt to low temperatures is crucial for future food security. While existing research has shed light on how certain C_4 grass species respond to cold, comprehensive studies across diverse lineages are limited. Our study aims to fill this gap by investigating how various C_4 grasses respond to and adapt to low temperatures, focusing on leaf anatomy, transcriptomics, and physiological responses.

In our leaf anatomy analysis, we observed interspecific adaptations in temperate grasses, characterised by small mesophyll area and reduced metaxylem cell size, as potential keys to maintaining photosynthetic efficiency and water transport under cold conditions. However, intraspecific leaf adaptations in *Alloteropsis semialata* revealed unique patterns, with genotypes from cooler regions developing large phloem areas, potentially aiding in sugar translocation under low temperatures. Transcriptomic comparisons between C_3 temperate and C_4 tropical species revealed shared and distinct gene expressions in response to cold, indicating potential common and unique adaptations among grass lineages. Finally, physiological experiments with *A. semialata* highlighted common photoprotective mechanisms across genotypes, regardless of their photosynthetic pathways or geographic origins. Our findings suggest that the photosynthetic type, rather than geographic origin, plays a significant role in determining the tolerance of grass species to low temperatures in high-light environments.

In conclusion, our study investigates various adaptations in C_4 grasses to cold climates. We found unique and consistent leaf anatomical adaptations among C_4 temperate grasses and observed shared and distinct gene expressions between C_3 and C_4 species. Our findings highlight the importance of photosynthetic type and geographic origin in determining how grasses tolerate low temperatures.

Chapter 1

General Introduction

1. C_4 photosynthesis

1.1 C_4 photosynthesis: what is it?

C_4 photosynthesis is a physiological adaptation that evolved from the ancestral C_3 type. This evolution was driven by a decrease in atmospheric CO_2 levels and an increase in seasonality, characterised by hot and dry periods, during the climatic changes of the Oligocene (23-33.9 million years ago) and subsequent Miocene period (5.3-23 million years ago) (Allmon, 2023). These environmental changes fostered the expansion of arid habitats, ultimately leading to the convergent evolution and diversification of various C_4 lineages.

Compared to C_3 photosynthesis, C_4 plants have modified their leaf anatomy and chemical processes allowing them to have higher photosynthetic efficiency by increasing the uptake of carbon dioxide (CO_2) for a given use of water or nitrogen (Sage, 2001, 2016; Osborne et al., 2006; Christin et al., 2008). In terms of leaf anatomical structure, photosynthesis happens only in the mesophyll tissue in C_3 plants, whereas C_4 plants have modified distinctive leaf anatomy (Kranz anatomy) by developing an extra photosynthetic tissue called the bundle sheath, which is formed around the vascular tissue and located between that vascular and mesophyll tissues (Figure 1.1). The cooperation of these two distinctive tissues allows C_4 plants to modify their chemical processes by carrying out carbon fixation twice, also known as double carbon fixation. First, atmospheric CO_2 diffuses into mesophyll cells, where it is catalysed by carbonic anhydrase (CA) to form bicarbonate (HCO_3^-). Phosphoenolpyruvate carboxylase (PEPC) then binds the HCO_3^- and PEP, producing oxaloacetate or oxaloacetic acid (OAA), the first carbon fixation product with 4 carbons (C4). OAA is then rapidly converted to malate (MAL) or aspartate (Asp) before being transported to bundle sheath cells, where it is decarboxylated to release CO_2 to the Calvin-Benson cycle (Ludwig, 2016). RubisCo (Ribulose-1,5-bisphosphate carboxylase/oxygenase), the first step of the Calvin-Benson cycle, then catalyses CO_2 fixation a second time.

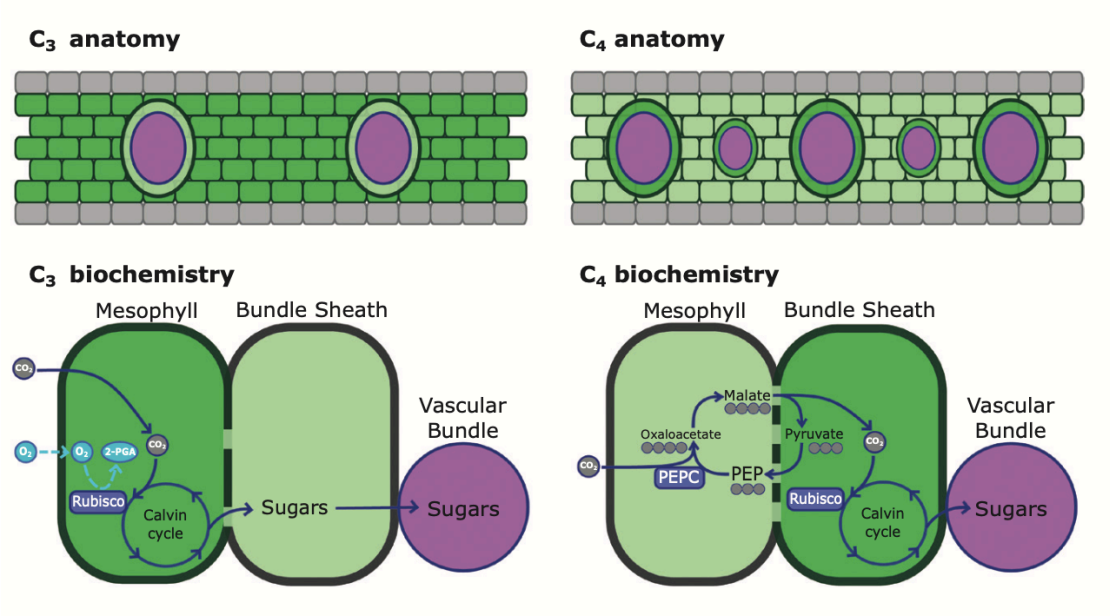

Figure 1.2 The comparison of leaf anatomy and biochemistry between C_3 and C_4 photosynthetic pathways. Colour: grey, epidermis cells; dark green, cells with many chloroplasts; light green, cells with fewer chloroplasts; purple, vascular tissue; dashed dark green line, mesophyll-bundle sheath interface; solid dark blue line, photosynthesis process; dashed light blue line, oxygenation (Young et al., 2020).

Historically, C_4 plants have been classified into three subtypes according to their major decarboxylation enzyme that releases CO_2 for the Calvin cycle: NADP-dependent malic enzyme (NADP-ME), NAD-dependent malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEPCK) (Hatch, 1987; Sage, 2004). However, model analysis of the "pure PEPCK subtype" indicates an imbalance of energy requirements in the bundle sheath and mesophyll cells (Wang et al., 2014). Thereby, NADP-ME and NAD-ME subtypes are suggested to be the distinctive C_4 photosynthetic pathways, with or without additional assistance from the PEPCK pathway (Wang et al., 2014; Rao et al., 2016).

Although RubisCo is the key enzyme of the Calvin cycle responsible for CO_2 fixation, it also has an oxygenase activity to catalyse oxygen (O_2) fixation, to form phosphoglycolate that is recycled in the photorespiratory pathway instead of continuing in the Calvin cycle (Peterhansel et al., 2010). Photorespiration is often viewed as a

wasteful pathway because it consumes energy, decreases photosynthetic efficiency, and results in the reduction of plant yield (Peterhansel et al., 2010; Busch, 2020). However, the CO_2-concentrating process in C_4 plants raises the CO_2 concentration in the bundle sheath cells, which is saturated enough to minimise O_2 fixation by RubisCo and therefore photorespiration. Thus, having double carbon fixation by using PEPC as an alternative enzyme for carbon fixation, and keeping RubisCo in the bundle sheath, are key modifications allowing C_4 plants to have a greater photosynthetic efficiency, and lead to higher yield production than the C_3 ancestral type.

1.2 C_4 photosynthesis and its geographical distribution

Several environmental factors play a crucial role in determining the geographical distribution of C_4 photosynthesis. One of the influential factors is light intensity. In conditions of high light intensity, C_4 plants exhibit accelerated growth rates, leading to greater yields compared to C_3 (Atkinson et al., 2016). Temperature is yet another vital factor, as C_4 plants exhibit a preference for warmer conditions . Furthermore, climatic variables such as precipitation and aridity levels are correlated with the abundance of C_4 species (Ehleringer et al., 1987). Lastly, seasonal variations influenced by precipitation patterns can impact the growth patterns of various plant types throughout the year (Ganjurjav et al., 2022).

Previous studies have demonstrated that various taxonomic categories of C_4 plants, such as Monocots (Poaceae, Cyperaceae) and Eudicots (Chenopodiaceae), exhibit distinct distribution patterns in response to climatic factors (Stowe et al., 1978; Pyankov et al., 2010). In Europe, the distribution of C_4 Chenopodiaceae is related to aridity and precipitation but not temperature, whereas the abundance of C_4 Monocots, Poaceae and Cyperaceae, shows a relation to temperature and aridity but not precipitation (Pyankov et al., 2010). In Australia and South America (Argentina), C_4 Poaceae are more abundant where the summer is hot and wet, and less abundant with decreasing temperature and/or decreasing summer rainfall (Hattersley, 1983; Cabido et al., 2008). Additionally, C_4 Poaceae from a subtropical monsoon forest in southern China are abundant in open habitats with a high-light environment (Ehleringer et al., 1987). Furthermore, the C_4 pathway, which provides an advantage in arid conditions, enables C_4 Poaceae to inhabit a broader range of drier habitats compared to their C_3

counterparts (Osborne et al., 2009). These findings point to a general geographical pattern for C_4 Poaceae: they tend to thrive in areas with high temperatures, humid summers, and arid conditions. However, it is worth noting that the evolution of cold tolerance in certain C_3 lineages should also be taken into account, as it has also impacted the composition of grassland communities worldwide (Edwards et al., 2010).

Furthermore, different decarboxylation pathways can also influence distribution patterns. For example, in Namibia, NADP-ME-type C_4 grasses occur mainly in wet areas, while the PEPCK-type dominates in the area of intermediate precipitation and the NAD-ME-type is abundant in the arid area (Schulze et al., 1996). However, the geographic patterns of C_4 grasses may be influenced by the characteristics of each species apart from the decarboxylation types (Cabido et al., 2008).

2. Poaceae, the grass family, as a model system for this study

2.1 The grass family, Poaceae, dominates C_4 photosynthesis

Among plants employing C_4 photosynthesis, the grass family Poaceae is most prevalent. Roughly 60% of all C_4 species belong to this family, which include major crops (maize, sugarcane), second-generation biofuels (miscanthus, switchgrass), and herbaceous species of tropical savannas (Sage et al., 1999).

The grass family (Poaceae) is a large plant family consisting of over 11,000 species. In terms of phylogeny, it can be categorised into two primary sister clades: PACMAD, which includes six subfamilies (Panicoideae, Aristidoideae, Chloridoideae, Micrairoideae, Arundinoideae, and Danthonioideae) (Sánchez-Ken et al., 2007; Bouchenak-Khelladi et al., 2008), and BOP, comprising three subfamilies (Bambusoideae, Oryzoideae, and Pooideae) (Soreng et al., 2022). The majority of C_3 grasses, about 57% of this family, belong to BOP, which diverged from PACMAD approximately 50 million years ago (Vicentini et al., 2008; Christin et al., 2008). Conversely, C_4 species constitute about 42% of this family, and together with their C_3 relatives are confined to the PACMAD clade (Osborne et al., 2014).

2.2 *Alloteropsis semialata:* a model plant to study photosynthetic pathway evolution and climatic adaptation

Alloteropsis semialata, also known as black seed or cockatoo grass, is a species belonging to the tribe Paniceae and the subfamily Panicoideae. It has intraspecific variation in photosynthetic processes and includes C_4, C_3-C_4 intermediate, and C_3 types (Lundgren et al., 2016). This diversity makes it an ideal system for examining the evolutionary connections between C_3 and C_4 traits.

A. semialata is native to the African continent and it is also found in Asia and Australia (Pereira et al., 2023). It commonly thrives in the tropics and subtropics across different geographic regions, each characterised by mean annual temperatures (MAT) ranging from 15 to 25 °C and mean annual precipitation (MAP) ranging from 500 to 2000 mm (Fick et al., 2017). This indicates that *A. semialata* is able to tolerate a wide range of different environmental conditions and temperature ranges across its natural habitat.

3. Low temperature as a factor limiting plant yield

Among various environmental factors, temperature is one of the major factors that has a wide impact on the productivity and geographical distribution of plants on a global scale. Living in suboptimal temperatures, too high or too low, may result in a decrease in yield production. In this study, we specifically investigate the effects of low temperatures. Temperatures ranging from 0 to 15 °C are defined as chilling or non-freezing temperatures (Theocharis et al., 2012), while temperatures below the freezing point of water (0 °C) are defined as freezing temperatures.

3.1 Effects of low temperatures on plants

Low temperatures can cause various effects on plants. In general, as temperature drops, plant physiology starts to change due to a slowdown in biochemical processes. These can cause a deterioration of plant performance or damage cell structures leading to cell death. However, these effects may be different in each species depending on their ability to tolerate the cold and the exposure time to low temperatures.

3.1.1 Changes in cell membrane fluidity and permeability

After being exposed to a chilling temperature, various biochemical and physiological cell functions are disrupted, causing visible injuries to plants including wilting, chlorosis, or necrosis (Ruelland et al., 2010). These often result from the alteration in cell membrane structure and lipid composition (Uemura et al., 1999). Membranes are the primary site of cold-induced injury, inducing a cascade of cellular processes with adverse effects on the plant. When exposed to the cold, the phase of membranes transitions from liquid-crystalline to solid gel. This causes a disruption in protoplasmic streaming (cyclosis), interrupted activation of energy-bound enzymes, and increased membrane permeability. Consequently, electrolytes and ions leak from the cell, resulting in imbalances in ion contents and metabolites, and causing the accumulation of toxic metabolites in cells. Additionally, low temperatures trigger an excess intracellular generation of reactive oxygen species (ROS), which includes singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) formation. Moreover, low temperatures disrupt the antioxidant system to exacerbate photooxidative stress. This stress leads to lipid peroxidation and membrane damage (Zhang et al., 2019). If the exposure is no longer than a threshold for each species, the plant will recover and survive. However, if exposure is maintained, plant cells will be damaged, resulting in necrosis and death (reviewed in Theocharis, Clément and Barka (2012)).

3.1.2 Changes in cell wall rigidity

Although the plasma membrane is the main site of damage by low temperatures, several studies reveal that the cell wall is an integral barrier to ice nucleation and propagation, which acts to reduce freezing damage to membranes (Takahashi et al., 2021). However, the function of cell walls is modified at low temperatures. A study in *Arabidopsis thaliana* grew plants at 4 ºC for 12 to 48 h showing the down-regulation cell-wall genes that promote cell expansion (Hannah et al., 2005). Moreover, in the same species, upregulation of genes involved in the accumulation of matrix polysaccharides and wall-remodelling enzymes occurred after prolonged chilling at 4 ºC, resulting in enhanced or reduced cell-wall rigidity depending on the tissue (Panter et al., 2020).

3.1.3 Decrease in photosynthetic efficiency

Photosynthesis is interrupted at low temperatures due to decreases in gene expression and enzyme activity. RubisCo is a key enzyme in the Calvin cycle involved in CO_2 fixation for C_3 and C_4 plants. At low temperatures, RubisCo becomes more limiting for C_4 photosynthesis (Kubien et al., 2003). In various non-cold acclimated or cold-sensitive plants the genes encoding for RubisCo and RubisCo activase are downregulated, resulting in decreases in their content and photosynthetic efficiency when plants are grown under chilling temperatures. For instance, in a study by Wang et al. (2008), chilling-sensitive maize showed a 40% loss of RubisCo content after 9 days of chilling at 14 °C. Moreover, PEPC also shows the same trend of decreasing gene expression, enzyme content and activity (Wang et al., 2008; Long et al., 2013).

While photosynthetic efficiency decreases due to the impact of low temperatures, plants are still exposed to the same light intensity, which may be high during the daytime. The excess light energy that is not used for photosynthesis decreases the rate of the photosynthetic electron transport chain (PETC) through a phenomenon called photoinhibition. The extent of photoinhibition results from an imbalance between the rate of photodamage of photosystem II (PSII) and its repair. The photodamage occurs because strong light generates free radicals from the PETC, inducing the production of ROS in the cell, leading to an inactivation of the photochemical reaction centre of PSII followed by a decrease in PETC (Fryer et al., 1998). Low temperature itself not only causes the overgeneration of ROS, but also inhibits the repair of PSII by disrupting the synthesis of the D1 protein precursor, which is needed for the assembly of the active PSII complex and the repair of PSII (Nath et al., 2013).

Chlorophyll is another important factor that has a direct impact on photosynthetic capacity. In photosynthesis, chlorophyll is a pigment essential for harvesting and transducing light energy in the antenna systems of chloroplasts, and for mediating charge separation and electron transport in reaction centres (Cardona et al., 2012) At low temperatures, chlorophyll content decreases, which is caused by the inhibition of chlorophyll biosynthesis due to the suppression of gene expression and activities of biosynthetic enzymes (Zhao et al., 2020; Wang et al., 2021). This response

results in the yellowing of the leaf, also known as chlorosis, at low temperatures (Wang et al., 2021).

3.1.4 Decrease in sugar translocation in the phloem

Sugar translocation is the process by which sugars, primarily sucrose, are moved from the source tissues (where they are produced) to the sink tissues (where they are stored or utilised). This process is bidirectional, allowing sugars to move in both directions, from source to sink and vice versa. This process relies on a pressure-driven mechanism called pressure flow, which is related to the process of sugar loading/unloading, water movement, and pressure building up in phloem vessels (Knoblauch et al., 2016).

At low temperatures, the rate of phloem flow and sugar loading from leaves to the phloem decreases. This can result from a rise in the viscosity of sugar solution in the phloem sap (Telis et al., 2007). In addition, in species which load the phloem using an active transport mechanism, that process would be limited by low temperatures because of the slow-down of ATP synthesis (Zhang et al., 2018). Another factor affecting the movement of assimilates from source to sink tissues is the impairment of crucial proteins responsible for sugar loading, including Sucrose Transporters (SUTs) (Takahashi et al., 2017) and Sugars Will Eventually be Exported Transporters (SWEETs) (Gautam et al., 2022).

3.1.5 Disruption in water transportation

Low temperatures can decrease the absorption and movement of water in plants. This is caused by decreased permeability at the root membrane together with the increased viscosity of water (Jensen et al., 1961). Moreover, another phenomenon disrupting water movement through xylem vessels is ‘freeze-thaw-induced embolism’. According to the ‘thaw-expansion hypothesis’ (Mayr et al., 2010), the mechanism consists of two main steps. First, below the freezing point of water (< 0 °C), ice formation initially occurs in extracellular spaces. Subsequently, ice formation expands, increasing pressure within the xylem vessels and surrounding tissues, which can cause mechanical tension on the cell wall and other structures. Additionally, the expansion increases the solute concentration outside the cells, leading to a decrease in water

potential. This drives water out from the cells to equalise the water potential causing cellular dehydration. The pressure from ice formation consequently causes air bubbles to form within the xylem vessels. Second, when temperatures rise and the ice begins to thaw, the water within the vessels transitions from solid back to liquid, leading to pressure changes within the vessels. This pressure potentially forces the air bubbles to integrate and further expand if the pressure becomes sufficiently negative to counter the bubble-collapsing force of surface tension.

3.1.6 Decrease in cellular respiration

Cellular respiration in plant cells is closely tied to carbohydrate metabolism. It involves the utilisation of stored carbohydrates, which are products of photosynthesis, to generate essential forms of energy, including adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NADH), and flavin adenine dinucleotide ($FADH_2$). These energy-rich compounds energise various vital biochemical processes and facilitate active transportation between cells.

Numerous studies have demonstrated that lower temperatures lead to a decrease in the rate of cellular respiration. For instance, in potato tubers (*Solanum tuberosum*), a chilling environment (0-10 °C) results in a notable reduction in respiration rates across various plant tissues (ap Rees et al., 1988). Similarly, in barley leaves, the respiratory rate measured through oxygen consumption during darkness is roughly half at 5 °C compared to that at 25 °C (Labate et al., 1989a). This decline in respiration leads to reduced production of ATP, causing a slowdown in critical cellular processes that require energy, such as apoplastic phloem loading.

3.2 Mechanisms of acclimation to low temperatures

To deal with the effects of exposure to low temperatures, plants need to induce alterations to gene expression which then trigger biochemical and physiological modifications to enhance their tolerance and prevent damage. This phenomenon is known as chilling or cold acclimation (Smallwood et al., 2002; Theocharis et al., 2012).

3.2.1 Modification in plasma membranes and cell walls

Phospholipids are the main component of plasma membranes, and chloroplast and mitochondrial envelopes. The fluidity of these structures depends on the ratio of saturated and unsaturated fatty acids. A greater unsaturated fatty acid content increases membrane fluidity and depresses the threshold temperature for membrane damage (Uemura et al., 1999). Cold-acclimated plants therefore have higher unsaturated fatty acid contents in their membranes relative to non-acclimated plants (Campos et al., 2003; Cruz et al., 2010; Tian et al., 2022).

Cold-acclimated plants may alter cell wall ultrastructure, as well as modify membranes. In cold-acclimated grapes (*Vitis* spp.) and apples (*Malus domestica*), cell-wall strength increases, whereas cell-wall pore size decreases (Rajashekar et al., 1996). Furthermore, the cell walls of winter oilseed rape (*Brassica napus* L. var. *oleifera* L.) are thickened after acclimation at 2 °C (Stefaniwska et al., 2002). Additionally, low temperatures can induce phenylalanine ammonia-lyase (PAL) activity, which is associated with the accumulation of phenolic compounds in the leaf and also the modification of the cell ultrastructure (Stefaniwska et al., 2002).

3.2.2 Photosynthetic acclimation

Photosynthesis is particularly vulnerable to low temperatures since light interception remains unchanged, while the metabolic capacity to utilise light energy is impaired. To maintain photosynthetic efficiency at low temperatures, cold-acclimated plants therefore need to change their characteristics at various biological levels, ranging from biochemical to anatomical and physiological.

3.2.2.1 Biochemical adaptations

Various cold-acclimated and cold-tolerant species, including grasses, demonstrate the maintenance and/or increase in gene expression, as well as the amount and activity of photosynthetic enzymes such as RubisCo and PPDK, which are rate-limiting for photosynthesis. For instance, *Miscanthus* × *giganteus*, a cold-tolerant grass, exhibits elevated levels of RubisCo or PPDK enzymes to sustain photosynthesis under low temperatures (Long et al., 2013). Additionally, to reduce photodamage caused by excess light when plants have lower photosynthetic activity, the repair of PSII is

necessary. In cold-hardened leaves of winter rye (*Secale cereale L.*), the capacity for synthesis of the D1 protein and catalase is increased to repair PSII by inducing the assembly of the PSII complex (Shang et al., 2003). Moreover, plants may increase their chlorophyll content in response to lower temperatures, which may help plants to compensate for photosynthetic efficiency. For instance, all leaves of winter rye grown at 6 °C exhibit increased chlorophyll content per leaf (Griffith et al., 1989).

3.2.2.2 Morphological and anatomical adaptations

Some cold-acclimated plants show changes in their leaf structures to enhance their photosynthetic efficiency. The main sites of photosynthesis in C_4 species are the bundle sheath and mesophyll tissues, which contain chloroplasts. Hence, the alteration in those tissues will have an impact on photosynthetic capacity.

The RubisCo enzyme, primarily found in bundle sheath cells, is a significant factor limiting C_4 photosynthesis at low temperatures. This leads to the expectation that C_4 plants will adapt to low temperatures by enlarging the RubisCo-containing bundle sheath cells (Kubien et al., 2004).

3.2.2.3 Physiological adaptations

To protect plants from photoinhibition and photodamage, acclimated plants enhance non-photochemical quenching (NPQ), which is a protective mechanism that dissipates excess absorbed light energy as heat. NPQ operates within the photosynthetic membranes of plants, as described by Ruban (2016). In addition to NPQ, plants employ various mechanisms, including non-regulated processes (NO), to release excess light energy. NO processes encompass biochemical pathways or physiological responses that occur independently of regulatory mechanisms, such as hormonal signalling or environmental effects. While these processes play essential roles in plant growth, development, and metabolism, they may also produce harmful by-products that can inhibit photosynthesis (Kuhlgert et al., 2016).

Protective mechanisms against low temperatures often manifest in changes in plant leaf colouration due to pigment accumulation. For example, anthocyanin accumulation occurs in species like *Pinus banksiana* (jack pine) (Huner et al., 1998) during winter, imparting a purple hue to leaves and shielding them against PSII

photoinhibition by screening irradiance. Similarly, *Thuja plicata* (western red cedar) seedlings acclimated to low temperatures exhibit red-brown leaves attributed to increased carotenoid levels, which serve as photoprotective pigments. However, this colouration tends to diminish a few days after transferring back to normal temperatures (Weber et al., 2003).

3.2.3 Accumulation of ROS and activation of scavenging system

To reduce the excess ROS production triggered by low temperatures, acclimated plants respond by activating antioxidant systems to mitigate oxidative damage. Plants may increase the production of low-molecular-weight antioxidants, such as ascorbic acid (Asc), glutathione (GSH), tocochromanols and reduced prenylquinones. Moreover, they may activate and increase antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), peroxidase (POX), glutathione reductase (GR), glutathione peroxidase (GPX), and peroxiredoxins (PRXs). Each antioxidant and enzyme has its own specific mechanism to scavenge ROS, reviewed in Szymańska *et al.* (2017).

3.2.4 Maintaining water transport in xylem vessels

Water is the primary solvent within the cytosol, serving as the medium for various crucial biochemical processes within a cell. Consequently, any disruption in water transport through xylem vessels can lead to osmotic imbalances, resulting in cellular wilting and a deceleration of metabolic processes. Notably, studies by Brodribb and Feild (2000) have demonstrated a positive correlation between xylem hydraulic conductance and the maximum quantum yield, emphasising the importance of maintaining water transport in the xylem for efficient photosynthesis, especially in low-temperature conditions.

In frigid winter climates, plants confront the challenge of ‘freeze-thaw-induced embolism’. Wider xylem vessels, characterised by a greater abundance of pits, are more susceptible to embolism due to their capacity to facilitate an air-bubble expansion (Carluccio et al., 2023). To counteract this, plants in temperate and cold regions, such as conifers (Davis et al., 1999; Pittermann et al., 2006) and barley (*Hordeum vulgare*) (Tamang et al., 2022), have evolved to reduce the diameter of their xylem vessels. This

adaptation serves to increase hydraulic pressure within the vessels, requiring less time and water to refill compared to wider conduits (Vesala et al., 2003). Consequently, plants with smaller vessels are better equipped to rapidly recover from embolism, ensuring the maintenance of a functional xylem water transport network while thriving at low temperatures.

3.2.5 Maintaining sugar translocation in phloem

Sugar translocation in phloem is a fundamental process that ensures the efficient allocation of resources within a plant, allowing it to adapt and thrive in diverse environmental conditions. It is a key factor in the ability of the plant to grow, reproduce, and respond to its surroundings. Specifically, in response to low-temperature stress, plants that have undergone cold acclimation exhibit adaptations in their phloem structures and phloem-loading mechanisms, ensuring the sustained functionality of this vital process.

One significant aspect of phloem structural adaptations involves sieve pores (SPo). These specialised structures are located on a sieve plate connecting sieve elements (SEs) and also between SEs and companion cells (CCs) (Kalmbach et al., 2019), indicating that they play a dual role in both transporting materials through SEs and facilitating sugar loading/unloading via CCs. In high-altitude conifers, the key to successful adaptation lies in having a narrow and abundant number of SPo (Schröter et al., 2021). These narrow pores create a large surface area compared to their volume, while having plenty of them ensures a backup system, reducing the chance of blockages in nutrient flow. These adaptations help the plant quickly and effectively move sugars and other important molecules around.

The phloem-loading mode in plants is also significantly influenced by temperature. Symplastic loaders tend to be more sensitive to cold compared to apoplastic loaders (Gamalei, 1989; Van Bel et al., 1992). The symplastic loading pathway appears to be particularly inhibited at low temperatures (Van Bel, 1993). Additionally, plants that live in cold and dry climates typically exhibit lower plasmodesmatal frequencies in the phloem of minor veins, indicating a preference for the apoplastic pathway, which relies less on transportation through plasmodesmata than

the symplastic pathway (Van Bel et al., 1992; van Bel et al., 1992; Davis et al., 1999; Davidson et al., 2011).

4. Grass acclimation and adaptations to low temperatures

4.1 Previous studies: how grasses acclimate and adapt to survive at low temperatures

The Poaceae family, commonly known as grasses, represents a prominent group of plants that have successfully migrated to colder temperate regions (Hopkins et al., 2006; Linder et al., 2018). Among the 12 subfamilies within Poaceae, Pooideae emerges as a notable lineage, particularly prevalent in temperate and arctic regions (Schubert et al., 2019). Notably, members of this subfamily exclusively utilise C_3 photosynthesis. However, C_4 species have also repeatedly adapted to low-temperature environments.

C_4 grasses originate from tropical and subtropical regions, and they tend to thrive in humid and warm areas with high-light intensity environments (Hattersley, 1983; Ehleringer et al., 1987; Cabido et al., 2008). Interestingly, C_4 grass lineages have migrated more frequently into cold environments than their C_3 counterparts (Watcharamongkol et al., 2018) and there is evidence showing that numerous species have successfully migrated to grow in temperate and high-altitude regions, which are characterised by low temperatures and freezing events (Hopkins et al., 2006; Linder et al., 2018). For example, Spike Muhly (*Muhlenbergia glomerata*) (Kubien et al., 2004), *Cleistogenes squarrosa* (Liu et al., 2008), common cordgrass (*Spartina anglica*) (Long et al., 1975), and Bermuda grass (*Cynodon dactylon*) (Ahmad et al., 2016). In some C_4 grass crops, several genotypes may be cultivated at low temperatures such as miscanthus (*Miscanthus x giganteus*) (Wang et al., 2008; Farooq et al., 2009; Głowacka et al., 2014; Yin et al., 2016). These examples suggest that these C_4 grasses should have developed and evolved some mechanisms to prevent damage from low temperatures.

4.2 Leaf structural adaptations to low temperatures in grasses

Changes in leaf anatomy can exert significant influences on plant performance at both biochemical and physiological levels, particularly in the context of photosynthesis. Several studies have highlighted that various grass species have developed diverse leaf structures in different tissues to acclimate to low temperatures, potentially involving distinct adaptive mechanisms.

The main sites for C_4 photosynthesis are the mesophyll and bundle sheath (BS) cells. Importantly, the RubisCo enzyme, primarily found in chloroplasts of BS cells, is a major limiting factor for C_4 photosynthesis at low temperatures. As a result, C_4 grasses may adapt over time by increasing the size of RubisCo-containing BS cells (Kubien et al., 2004). This idea is supported by a study conducted by Bilska-Kos *et al.* (2018), which observed an enlargement of BS cells in cold-tolerant maize (*Z. mays*) after exposure to low temperatures. However, the study by Pignon *et al.* (2019) reveals that, despite structural differences among four C_4 grasses, the volume of bundle sheath chloroplasts does not increase in chilling-tolerant species compared to their sensitive counterparts. This indicates that the size of chloroplasts in bundle sheath cells may not be an inherent limitation for C_4 grasses to adapt to cold environments. Altogether, this suggests that the enlarged BS in C_4 plants under low temperatures may have different functions such as the import and export of nitrogen and sulphur, carbohydrate synthesis, metabolism of ROS, photorespiratory carbon metabolism, storage for water, ions, and carbohydrates, maintaining leaf hydraulic conductance and cavitation repair, and structural support (Leegood, 2008; Griffiths et al., 2013).

The water transport process in xylem vessels can be disrupted by freeze-thaw embolism. To counteract this, cold-adapted plants have developed smaller xylem vessels. For instance, *Cynodon dactylon*, a C_4 grass found in high-altitude regions with low temperatures, exhibits anatomical modifications that reduce the lumen area of individual metaxylem vessels (Ahmad et al., 2016). Similarly, the freezing-tolerant genotypes of winter barley (*Hordeum vulgare*) also possess a hereditary trait of small metaxylem vessel diameter (Tamang et al., 2022).

Research on how grasses maintain phloem transport at low temperatures is limited to a few species, and the underlying mechanism remains unclear. For instance, a study by Sowiński et al. (2001) demonstrated that chilling-tolerant maize has a less

frequent plasmodesmatal network within the phloem compared to chilling-sensitive maize. This suggests that in order to tolerate low temperatures, the plant relies more on apoplastic phloem loading than the symplastic loading pathway. Despite some insight into phloem anatomy adaptations to low temperatures, there is a lack of studies in various grass lineages, indicating the need for further research to elucidate the underlying mechanisms.

Based on earlier research (Watcharamongkol, 2019). it has been observed that certain temperate C_4 grasses possess lower leaf moisture content, which differs from many grass species found in tropical climates. The specific cellular factors contributing to this trait are still unclear. However, anatomical measurements in other species have indicated a strong correlation between cell size and tissue moisture content (Garnier et al., 1994), implying that lower leaf moisture content in temperate species might arise from smaller cells than in tropical species.

4.3 Biomolecular and transcriptomic adaptations to low temperatures in grasses

At the biomolecular level, responses to cold in C_4 grass species could be related to changes in gene expression and the properties and amounts of photosynthetic enzymes or PSII-associated proteins (Naidu et al., 2004; Long et al., 2013). For example, RubisCo and pyruvate-orthophosphate dikinase (PPDK) are thought to be rate-limiting steps of photosynthesis at low temperatures for the C_3 and C_4 biochemical cycles. The expression of both genes is increased in *M. x giganteus* under chilling conditions, while their expressions are impaired in *Z. mays* which is not typically a cold-tolerant plant (Long et al., 2013). Apart from that, *M. x giganteus* grown in chilling conditions significantly increases mRNA levels of several genes encoding PSII-associated proteins such as psbA (D1) and petA (Cytochrome F) (Spence et al., 2014).

4.4 Physiological adaptations to low temperatures in grasses

At the physiological level, the primary adaptation of C_4 plants is the ability to maintain the efficiency of photosynthesis, especially PSII. There are several studies on *M. x giganteus*, an especially cold-tolerant C_4 grass, showing that chlorophyll fluorescence parameters used in studies of PSII photochemistry remain unchanged after

exposure to low temperatures (Bilska-Kos et al., 2018). Moreover, *M. x giganteus* is able to recover light-saturated leaf CO_2 uptake (A_{sat}) to 90% of the pre-chill level within seven days after transferring to chilling conditions (Long et al., 2013).

To protect themselves from photodamage, various grasses, including both C_3 and C_4 photosynthesis types, use NPQ mechanisms to dissipate excess light energy. Examples include *Lolium perenne* and *Bromus inermis*, C_3 grasses, and *Bromus inermis* and *Pennisetum clandestinum*, C_4 grasses (Liu et al., 2008).

Moreover, some grasses exhibit the accumulation of anthocyanin in their leaves such as Rough bluegrass (*Poa trivialis L.*) growing under high-intensity light (Petrella et al., 2016) and *Zoysia japonica* (Zoysiagrass) (Jin et al., 2022) growing under low temperatures.

4.5 Adaptations to low temperatures in *A. semialata*

Alloteropsis semialata is a unique grass with diversity in photosynthetic pathways and adaptation to broad ranges of precipitation and temperature (Lundgren et al., 2016). C_4 plants of *A. semialata* have higher leaf growth and photosynthesis than the C_3 type at high temperatures. These decrease with temperature until 15 °C before being completely lost due to photoinhibition and photodamage (Osborne et al., 2008). However, the C_3 subspecies show an ability to acclimate to the cold after growing under chilling treatment for 14 days, which is not seen in the C_4 type. This implies that in lower temperatures, the C_3 variants of this species might outperform the C_4 due to their enhanced resistance to freezing damage, rather than their higher photosynthetic rates (Osborne et al., 2008). However, previous studies about how different genotypes of *A. semialata* respond to low temperatures have been limited to only two populations, representing C_3 and C_4 types, and there have been no studies about leaf structural adaptation in response to cold in this species.

5. Thesis aims and expectations

This thesis aims to investigate how different grass lineages respond to low temperatures and whether they have converged on the same or use different mechanisms of response at three different biological levels: leaf anatomy, gene expression, and physiology. The predominant focus of the work is on C_4 grasses, taking a comparative approach to evaluate the generality of mechanisms across multiple independently evolved lineages, and focusing on *A. semialata* to investigate diversification within the newly evolved C_4 type. The overview of this thesis is shown in Figure 1.3.

The first study (Chapter 2) focused on leaf structural adaptation to low temperatures in 26 grass lineages comprising tropical and temperate C_3 / C_4 grasses. There are three main aims of this study which are (1) to elucidate climatic effects on leaf cell size and tissue volumes, (2) to investigate the correlations between cell size, tissue volume and leaf moisture content, and (3) to test for relationships between cell size, tissue area and the maintenance of photosynthetic efficiency at low temperatures.

In Chapter 3, we studied intraspecific leaf anatomical adaptation in *A. semialata* to low temperatures. This study was based on the results showing leaf structural adaptation in grasses in the previous chapter (Chapter 2). We mainly aimed to determine if those specific adaptations were crucial for grasses to survive in cold climates and thus developed early during the migration from tropical into cooler environments, or if they evolved later to optimise physiological processes in cooler conditions. To address these questions, we have selected *A. semialata* as a newly evolved C_4 plant model with an ability to survive across broad temperature ranges. In this study, we hypothesised that intraspecific adaptations are an essential prerequisite for migration and will be the same as interspecific ones.

Chapter 4 of this thesis is about transcriptomic responses to short-term chilling temperatures in C_3 temperate and C_4 tropical grasses, focusing on adaptation mechanisms. In this study, we carried out leaf transcriptomic analysis of three grass lineages: *Beckmannia syzigachne* (BSY), a C_3 temperate Pooideae species, and two tropical C_4 species, namely *Cenchrus brownii* (CBR) from the subfamily Panicoideae and *Sporobolus consimilis* (SCO) from the subfamily Chloridoideae. We aim to elucidate how gene expression changes in grass leaves between the different lineages

responding to low temperatures and whether the same mechanisms are involved. Additionally, we further asked whether genes in C_4 tropical grasses shared common expressions among them and with a C_3 temperate species.

In Chapter 5 of this thesis, our focus was on studying photosynthesis acclimation and photoprotection in *A. semiala* under chilling and strong light conditions. For this investigation, we selected 12 distinct genotypes of *A. semialata* from diverse geographical locations, encompassing a range of photosynthetic types, including those exhibiting C_3-C_4 intermediate characteristics. The aim of this work was to see how *Alloteropsis* populations from different climate origins respond to low temperatures in terms of their photosynthesis efficiency and to test whether population differences arose from the extent to which they use non-photochemical quenching (NPQ) as a mechanism to protect them from photodamage. We further investigated how photodamage among these populations related to their photosynthetic type and geographical provenances.

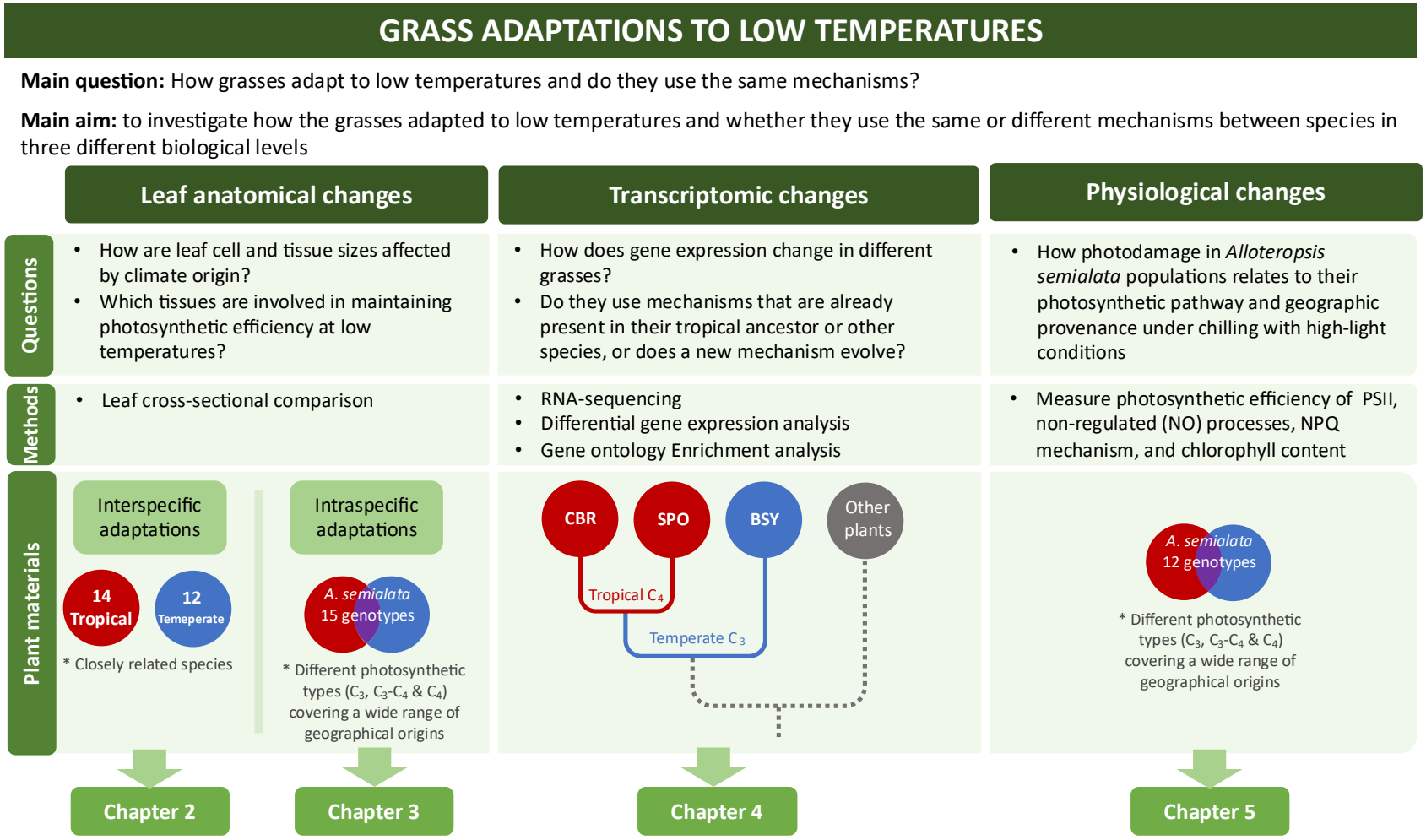

Figure 1.3 Overview of this thesis. For plant materials, the tropical and temperate climate origins of species are presented in red and blue, respectively.

Chapter 2

Interspecific leaf anatomical adaptation in C_4 grasses to tolerate chilling and freezing

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Personal contribution:

The study was co-designed by Colin Osborne, Pascal-Antoine Christin, and me. Alice Coppock, a former MRes student, prepared the leaf samples and microscope pictures, while Teera Watcharamongkol, a former PhD student, generated the datasets of cold and freezing tolerance in C_4 grasses. I extracted the quantitative traits from the microscope pictures using ImageJ, an image processing software. Lastly, I conducted all analyses, interpreted the results, and collaborated with all authors to write the paper.

1. Abstract

C_4 grasses originated from the tropical and subtropical regions, which have a high average temperature across the year. However, there is a lot of evidence showing that grasses can survive and grow in areas that experience low temperatures and that some C_4 species have adapted to tolerate chilling and freezing events. Although alterations in leaf anatomy in relation to cold tolerance have been observed in some C_4 grasses, few species have been assessed, limiting generalisation. In this study, we investigated the leaf anatomy of 14 tropical and 12 temperate grasses to identify those consistent changes involved in chilling and freezing tolerances. Our results show that temperate species have evolved reduced mesophyll tissues which may be related to the maintenance of photosynthetic and transport efficiency at chilling temperatures. Furthermore, our analysis revealed that the sizes of epidermis, mesophyll, and bulliform cells were positively correlated with moisture content, indicating that smaller cells in these tissue types could promote cold tolerance in C_4 grasses by reducing moisture content. Additionally, temperate species have reduced metaxylem vessel size compared to their tropical counterparts, which is expected to limit freeze-thaw embolism and maintain water transport and tissue hydration. Overall, our findings show that C_4 grasses share common interspecific leaf anatomical adaptations to low temperatures, which may be critical for maintaining photosynthesis and water transport in colder environments.

2. Introduction

C_4 photosynthesis is a physiological process resulting from a series of biochemical and anatomical modifications compared to the ancestral C_3 state, which together increase photosynthetic efficiency in warm and dry habitats (Hatch, 1987; Kellogg, 2013). About half of all C_4 species belong to the grass family (Poaceae) (Sage et al., 1999). C_4 grasses originated from tropical and subtropical regions, where the benefits of C_4 photosynthesis in terms of higher-efficiency biomass production and uses of light, water, and nitrogen are greatest (Long et al., 2013). However, recent work has shown that a number of C_4 grasses can survive across a broad temperature range, leading to the expansion of their niches into warmer regions and diversification into cooler environments (Watcharamongkol et al., 2018).

Living at low temperatures has significant impacts on various plant biochemical and physiological processes, especially photosynthesis (Theocharis et al., 2012; Szymańska et al., 2017). At low temperatures, photosynthesis is constrained by a reduction in activity of photosynthetic enzymes involved in CO_2 assimilation. For C_4 photosynthesis, the enzymatic rates of ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCo), phosphoenolpyruvate carboxylase (PEPC), and carbonic anhydrase (CA) restrict CO_2 assimilation (Boyd et al., 2015). CA catalyses the initial chemical step in the carbon-concentrating mechanism of C_4 plants (Studer et al., 2014) before the first carboxylation reaction by PEPC in the cytosol of mesophyll cells, followed by the second carboxylation in bundle sheath by RubisCo. Since RubisCo can catalyse oxygenation of ribulose bisphosphate (RuBP) requiring the energetically costly photorespiration pathway to avoid the accumulation of phosphoglycolate, having RubisCo in the bundle sheath has conferred superior photosynthetic efficiency in C_4 plants over their C_3 ancestors (Sage et al., 2013).

Low temperatures not only affect photosynthesis, but also disrupt transportation in plants. Vein tissues play a major role in transportation and consist of two types, xylem, and phloem, each with distinct functions. The xylem transports water and nutrient uptake from the root to the leaf and provides mechanical support and storage, while the phloem is responsible for sugar translocation from source to sink (Schulz et al., 2009; Sack et al., 2013). Both xylem and phloem consist of living and non-living

tissues. In living tissues, intracellular ice formation can cause cell damage, while extracellular ice formation can cause cellular dehydration, which mechanically damages cell membranes (Yamada et al., 2002). For non-living xylem conduits, freezing temperatures followed by thawing can cause cavitation or embolism resulting in the loss of xylem function and water-transport disruption (Sperry et al., 1992; Sevanto et al., 2012; Maruta et al., 2020). Based on the study by Jensen et al. (2013) the volumetric flow rate of phloem sap is dependent on the pressure differential driving the flow, the viscosity of the sugar solutions in phloem sap, and the shape and size of phloem cells. At low temperatures, the viscosity of the sugar solutions is increased, depending on the types of sugar present and their concentrations (Telis et al., 2007). Concurrently, low temperatures limit ATPase activity, which slows apoplastic sugar loading/unloading pathways (Giaquinta, 1979). Hence, to remain active during life at low temperatures, one of the solutions for plants is to evolve the xylem and phloem anatomy to compensate for the reduction and disruption of transportation.

There are several pieces of evidence already showing that the leaf anatomy of C_4 plants changes after transfer to low temperatures. For example, the bundle sheath (BS) cell size in a chilling-tolerant *Zea mays* accession increases after growth at low temperatures (Bilska-Kos et al., 2018). As the capacity of RubisCo is a major rate-limiting step during C_4 photosynthesis at cool temperatures, plants will need to compensate for the efficiency of photosynthesis by enlarging their RubisCo-containing BS cells (Kubien et al., 2003). Furthermore, *Cynodon dactylon*, a C_4 grass living in high-altitude regions experiencing low temperatures, shows anatomical alterations to reduce the lumen area of individual metaxylem vessels (Ahmad et al., 2016). Finally, according to previous work (Watcharamongkol, 2019), some temperate C_4 grasses have low leaf moisture content, which contrasts with many grass species from tropical climates. The cellular basis for this characteristic remains unknown, however, although anatomical measurements in other species have shown a close association between cell size and tissue moisture content (Garnier et al., 1994). In combination, these findings suggest that low leaf moisture content arising from small mesophyll cells or other tissue types, a large size of bundle sheath cells, and a small size of xylem vessels may promote freezing tolerance in C_4 grasses. However, the generality of these associations has not been tested.

To make generalisations about leaf anatomical adaptations to low temperatures among multiple C_4 grass lineages (interspecific adaptations), we compare the leaf anatomy of closely related pairs of 26 species originating from tropical and temperate climates. These quantitative data are used to test the effects of climate on the total volume and cell size of each tissue. We particularly tested whether cold-adapted plants have enlarged bundle sheath cells and reduced xylem vessel size compared with their tropical sister species. Furthermore, we also tested whether the composition of specific leaf tissues are responsible for the variation in leaf moisture underlying freezing tolerance. Overall, this work sheds new light on the leaf changes allowing the colonisation of colder climates by C_4 grasses.

3. Materials and methods

Plant material and environmental conditions

Leaf samples of 26 different C_3 and C_4 species that originate from tropical and temperate regions are used in this study (Table 2.1). The Köppen-Geiger classification was used for categorising climate types based on the coldest month temperature: tropical (average above 18 °C) and temperate (average between 0 and 18 °C). The samples were previously collected from plants grown to study the physiological response of C_4 grasses to cold treatments (Watcharamongkol, 2019). The leaf samples used in this leaf anatomical study were from the plants in the control treatment. In brief, the plants were grown in individual pots in a controlled environment chamber providing a 12 h photoperiod and a photon flux density (PFD) of 600 µmol m^{-2} s^{-1} measured at the plant height, day/night temperature of 25/20 °C, and relative humidity of 60/80% for 14 weeks, before leaves were collected.

Table 2.1 26 grass species sampling for this study

Species	Subfamily	Tribe	Subtribe	Type	Climate
Andropogon chinensis	Panicoideae	Andropogoneae	Andropogoninae	C_4	Tro
Andropogon gayanus	Panicoideae	Andropogoneae	Andropogoninae	C_4	Tro
Hyparrhenia rufa	Panicoideae	Andropogoneae	Andropogoninae	C_4	Tro
Schizachyrium scoparium	Panicoideae	Andropogoneae	Andropogoninae	C_4	Tem
Cymbopogon citratus	Panicoideae	Andropogoneae	Anthistiriinae	C_4	Tro
Sorghastrum nutans	Panicoideae	Andropogoneae	Anthistiriinae	C_4	Tem
Acroceras zizanioides	Panicoideae	Paniceae	Boivinellinae	C_4	Tro
Cenchrus brownii	Panicoideae	Paniceae	Cenchrinae	C_4	Tro
Pennisetum flaccidum	Panicoideae	Paniceae	Cenchrinae	C_4	Tem
Panicum phragmitoides	Panicoideae	Paniceae	Panicinae	C_4	Tro
Panicum virgatum	Panicoideae	Paniceae	Panicinae	C_4	Tem
Miscanthus giganteus	Panicoideae	Andropogoneae	Saccharinae	C_4	Tem
Miscanthus oligostachyus	Panicoideae	Andropogoneae	Saccharinae	C_4	Tem
Miscanthus sacchariflorus	Panicoideae	Andropogoneae	Saccharinae	C_4	Tem
Saccharum officinarum	Panicoideae	Andropogoneae	Saccharinae	C_4	Tro
Sorghum timorense	Panicoideae	Andropogoneae	Saccharinae	C_4	Tro
Eragrostis spectabilis	Chloridoideae	Eragrostidinae	Eragrostidinae	C_4	Tem
Muhlenbergia racemosa	Chloridoideae	Cynodonteae	Muhlenbergiinae	C_4	Tem
Pappophorum mucronulatum	Chloridoideae	Cynodonteae	Pappophorinae	C_4	Tro
Tridens flavus	Chloridoideae	Cynodonteae	Pappophorinae	C_4	Tem
Sporobolus consimilis	Chloridoideae	Zoysieae	Sporobolinae	C_4	Tro
Sporobolus pyramidalis	Chloridoideae	Zoysieae	Sporobolinae	C_4	Tro
Molinia caerulea	Arundinoideae	Molinieae	Molininae	C_3	Tem
Leersia hexandra	Ehrhartoideae	outgroup	Oryzinae	C_3	Tro
Leersia virginica	Ehrhartoideae	outgroup	Oryzinae	C_3	Tem
Oxytenanthera abyssinica	Bamusoideae	outgroup	Bambusinae	C_3	Tro

Note: Abbv.: Tem, Temperate; Tro, Tropical

Investigation into the anatomical and structural changes in C_4 grass leaves

Slides of each C_4 grass leaf were prepared by Alice Coppock, a former MRes student. Briefly, one leaf, which is the third leaf from the top of each plant, were collected from each species. The cross-sectional leaf samples were harvested on either side of the leaf midrib at the middle of the leaf and fixed in 4:1 ethanol:acetic acid, embedded in a methacrylate embedding resin, cut in cross-sections, and stained by Toluidine Blue. The slides were photographed with a camera under a light microscope with 100x magnification.

To measure leaf tissue area, we focused on one leaf cross-sectional area per slide per species, specifically located between two adjacent secondary veins, defining this area as the selected cross-sectional area ($Area_{selected}$). The areas of five tissue types within the $Area_{selected}$ — epidermis (E), bulliform cells (BC), bundle sheath (BS), minor vein (MV), and mesophyll (M) (Figure 2.1) — were measured using ImageJ, were measured with ImageJ (Schneider, Rasband and Eliceiri, 2012).

To access the traits of vascular bundle tissue, we focused on all minor vascular bundles present within the $Area_{selected}$ of each species. The areas of phloem (Ph), xylem (Xy), and bundle sheath (BS) in these minor vascular bundles were measured (Figure 2.2 (A)). All cells presented in all the bundles were counted and calculated for means of particular tissue types.

Furthermore, we measured the cell sizes within each tissue type in the leaf, including epidermis (E), mesophyll (M), bundle sheath (BS), and bulliform cells (BC) that were located within the $Area_{selected}$. For the measurement of metaxylem vessel size, all the biggest xylem vessels that are in the major veins (secondary veins) existing in a leaf cross-section were chosen. Every cell was measured using ImageJ, but cells with an unclear border were excluded. The maximum cell length (MCL) is the longest dimension from one cell border including cell wall to another opposite end. Moreover, the maximum protoplast length (MPL or maximum lumen length (MLL) in case of the xylem measurement) was measured (Figure 2.2 (B)) and used for a calculation of mean cell wall thickness (CW_{thick}) by using the Equation 2.1.

$$CW_{thick} = \frac{MCL-(MPL\ or\ MLL)}{2} \qquad \text{Equation 2.1}$$

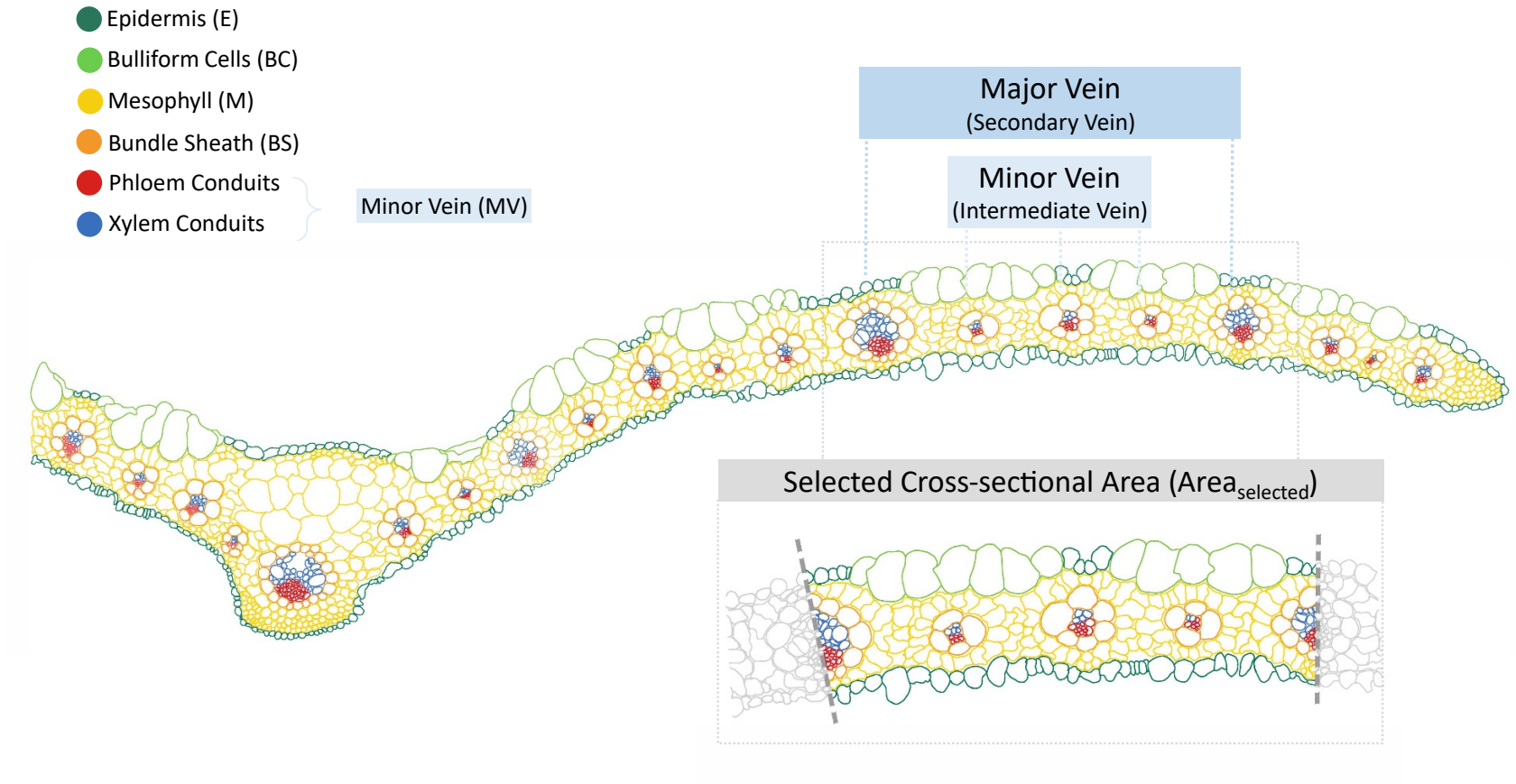

Figure 2.1 Measurement of leaf tissue area in a cross-section

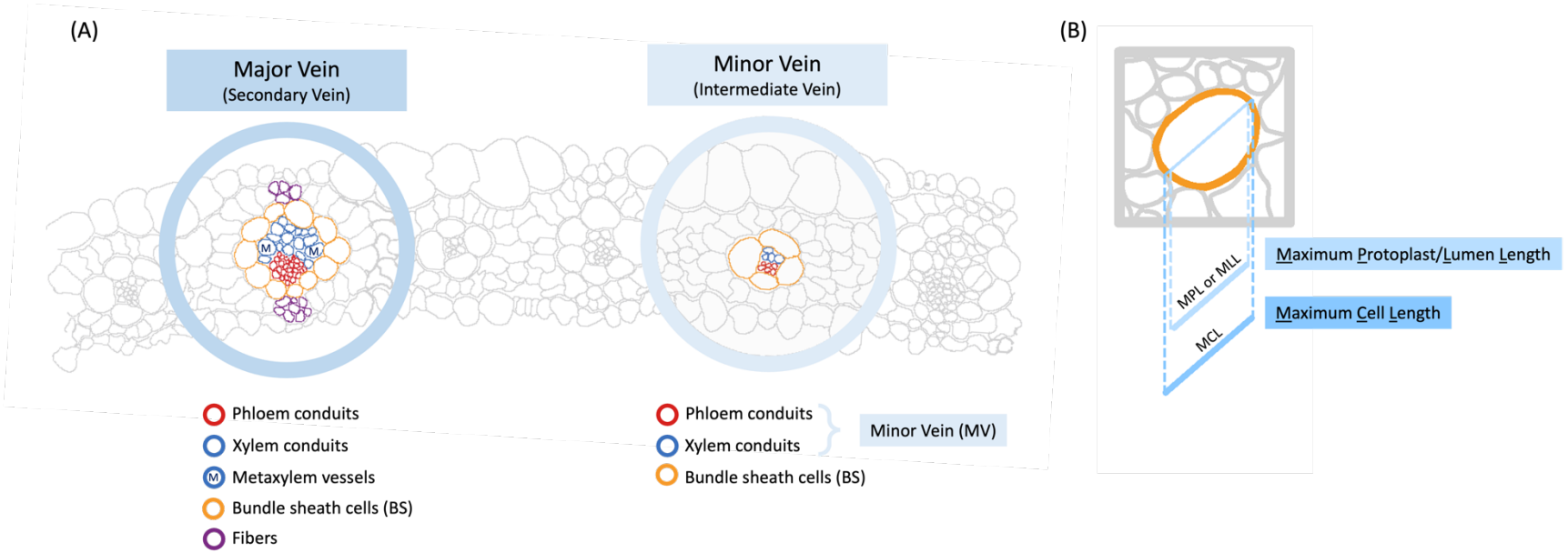

Figure 2.2 Diagrams of (A) veins in a cross-sectional image and (B) the measurement of cell size.

Maximum quantum yield of photosystem II (F_v/F_m)

The data describing photosynthetic efficiency were obtained from Chapter 3 of Watcharamongkol (2019). Briefly, the measurements for both a chilling treatment and a controlled environment (day/night temperatures of 15/5 °C and 25/20 °C, respectively) were done on day 1, 7, and 14 during the chilling phase, and then day 15, which was one day after freezing treatment. The freezing treatment was applied to all plants on the 15th day by gradually decreasing temperature from 15 to -5 °C overnight. Before a measurement, a mature leaf of each species was dark-adapted for 30 minutes. The minimum fluorescence yield following dark adaptation (F_0) and the maximum fluorescence yield following dark adaptation (F_m) were measured at room temperature using a PAM chlorophyll fluorometer (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany). The maximum quantum yield of photosystem II (F_v/F_m) was calculated by Equation 2.2.

$$Fv/Fm = \frac{F_m - F_0}{F_m} \qquad \text{Equation 2.2}$$

Additionally, the percentage change of F_v/F_m caused by the treatment was obtained from Equation 2.3 for further analysis.

$$\%\Delta F_v/F_m = \frac{(F_v/F_m \; after \; treatment) - (F_v/F_m \; control)}{(F_v/F_m \; control)} \times 100 \qquad \text{Equation 2.3}$$

Leaf moisture content

This data was obtained from a previous study, Watcharamongkol (2019). Briefly, nine mature leaves from each species that grew under the control treatment (day/night temperature of 25/20 °C) were collected and weighed for fresh mass (M_f) immediately and dry mass (M_d) after drying at 70 °C for 3 days. Leaf moisture content (MC) was calculated by the Equation 2.4:

$$MC = \frac{M_f - M_d}{M_d} \times 100 \qquad \text{Equation 2.4}$$

Statistical analyses

The effects of climatic types on the leaf tissue area and cell size of each tissue type were tested with a phylogenetic generalized linear regression model (PGLS). The published phylogenetic tree in Spriggs et al. (2014) and the Caper package in R were used in this analysis. The climatic types were used as a predictor and the ratio of tissue area to the $Area_{selected}$ and the maximum cell length (MCL) and cell wall thickness (CW_{thick}) of each tissue type were used as response variables.

To test for a relationship between leaf moisture contents and tissue areas and the relationship between moisture contents and cell sizes, a PGLS was also used. The ratio of tissue area to the $Area_{selected}$, and the MCL of each tissue type, were each used as a predictor, with leaf moisture content as the response variable.

Furthermore, PGLS was also used to investigate the correlation of leaf anatomy traits and the chilling and freezing responses of each species. The chilling responses are represented by the maximum quantum yield of PSII on the 7^{th} ($\%\Delta F_v/F_m$ AC7) and 14^{th} ($\%\Delta F_v/F_m$ AC14) days of the treatment, while the change of maximum quantum yield in PSII after freezing ($\%\Delta F_v/F_m$ AF) were shown as the effect of freezing responses.

4. Results

Climate effects on leaf anatomy traits; tissue areas and cell size

To examine the climatic effect on leaf tissue area, we use PGLS analysis to compare the areas of five tissue types between tropical and temperate grasses. The results indicate that only mesophyll (M) showed a weak statistically significant difference between tropical and temperate species with a p-value of 0.088 (Figure 2.3 and Table 2.2). However, bulliform cells (BC), bundle sheaths (BS), epidermis (E), and minor veins (MV) did not show statistical differences among tropical and temperate grasses (Figure 2.3 and Table 2.2).

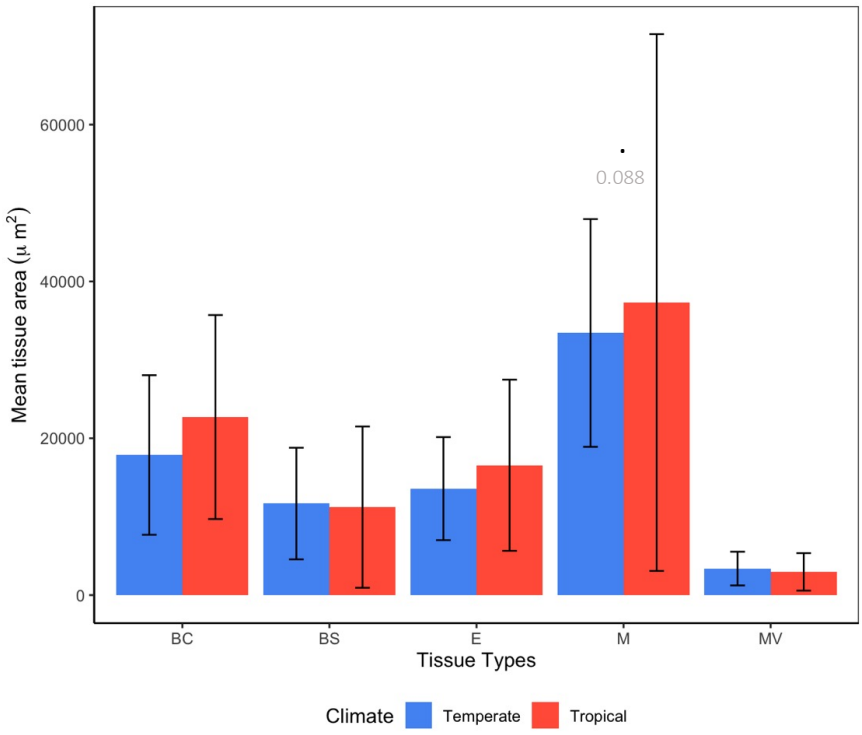

Figure 2.3 The PGLS analysis testing for climate effects on five leaf tissue areas (μm^2). Abbreviation: bulliform cells (BC), bundle sheath (BS), epidermis (E), mesophyll (M), and minor vein (MV). The error bars represent the variation in the mean ratio of tissue area and area selected within the tropical or temperate species. Significance level: ·, $p < 0.1$.

The temperate and tropical grass species in this study are distributed among five subfamilies, with all species in Chloridoideae and Panicoideae using the C_4 photosynthetic type (Figure 2.4). These species exhibit variation in BS and MV areas. Within these two C_4 subfamilies, *Acroceras zizanioides* from Panicoideae and *Sporobolus consimilis* from Chloridoideae show significantly larger BS and MV areas compared to other tropical grasses. Meanwhile, *Schizachyrium scoparium* from Panicoideae has the largest BS and MV areas among temperate species.

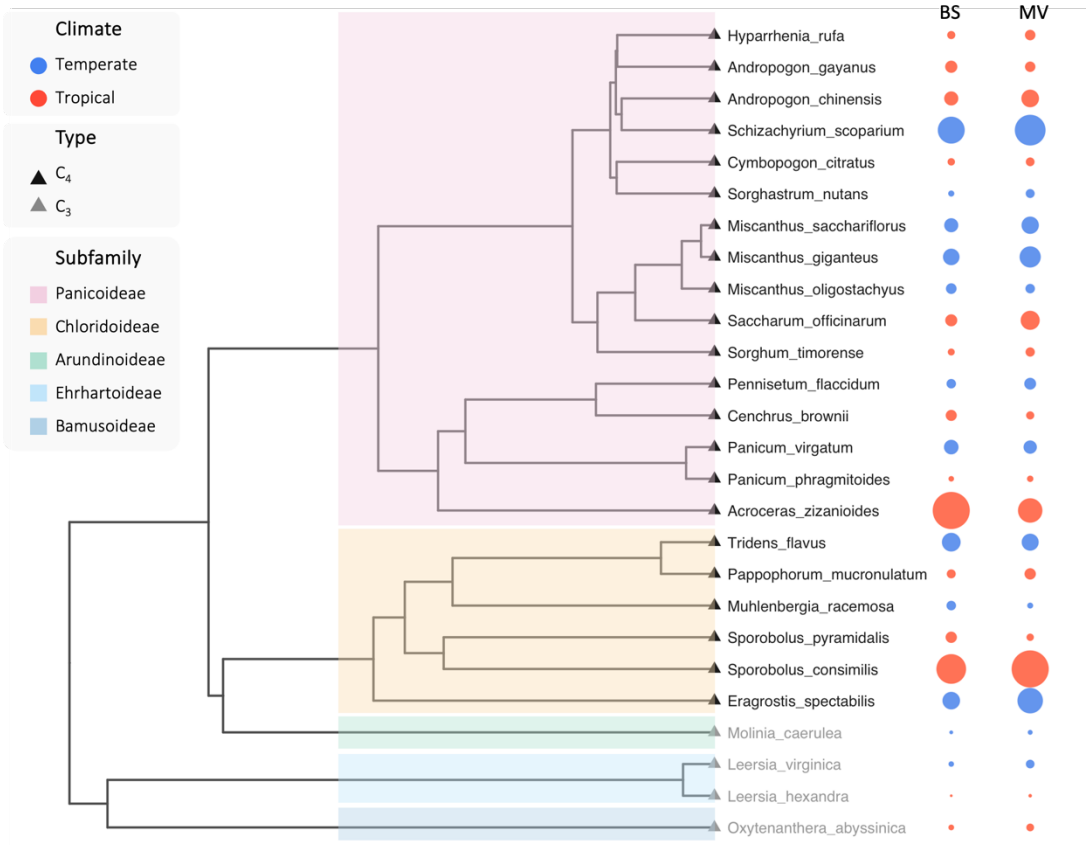

Figure 2.4 Grass phylogenetic tree showing climate effects on bundle sheath (BS) and minor vein (MV) cross-sectional areas of each species. The size of the dots represents the tissue area of each particular species.

We have further tested the climate effect on cell size from various tissue types by comparing mean maximum cell length (MCL) and mean cell wall thickness (CW_{thick}) of each cell type between temperate and tropical grasses. Due to the limitation of the image resolution, we were unable to measure CW_{thick} for any of the cell types except metaxylem. Our PGLS analysis showed that metaxylem vessels (MXy) have smaller MCL and thinner CW_{thick} in temperate than tropical species with p-values of 0.095 and 0.0006, respectively (Figure 2.5 and Table 2.2). The MCL of bulliform cell, bundle sheath, epidermis and mesophyll tend to be smaller in temperate than tropical lineages; however, there are no statistical differences among them. This result suggests that temperate species have smaller metaxylem vessel size and tend to have smaller cell size than their tropical counterparts.

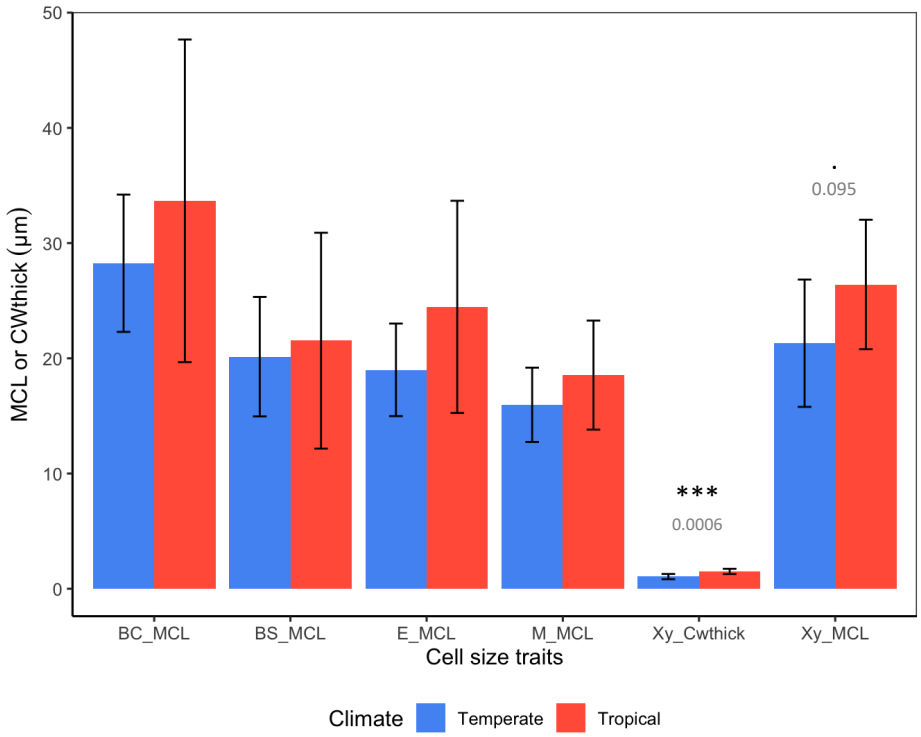

Figure 2.5 The PGLS analysis testing for climate effects on cell size of five different tissue types. Size of cells was represented by the maximum cell length (MCL) (µm) and mean cell wall thickness (CW_{thick}) (µm). Abbreviation: bulliform cells (BC), bundle sheath (BS), epidermis (E), mesophyll (M), and minor vein (MV). Significance level: ·, $p < 0.1$ and ***, $p < 0.001$.

Table 2.2 Results of PGLS analysis testing for the effects of climate types on the leaf cross-sectional tissue area and mean absolute cell size of each tissue type.

Variables	n	λ	Tropical species	Temperate species	p-value
Tissue area (μm²)					
BC	24	0.000	22704 ± 13006	17874 ± 10176	0.308
BS	24	0.000	11217 ± 10283	11675 ± 7117	0.899
E	24	0.000	16557 ± 10915	13575 ± 6571	0.417
M	24	0.990	37309 ± 34224	33432 ± 14523	0.088 ·
MV	24	0.000	2961 ± 2391	3386 ± 2149	0.640
Xy	16	0.000	584 ± 689	316 ± 290	0.671
Ph	16	0.000	265 ± 175	223 ± 76	0.792
MCL (μm)					
BC	26	0.880	33.669 ± 13.999	28.251 ± 5.958	0.593
BS	26	1.000	21.530 ± 9.368	20.143 ± 5.184	0.994
E	26	0.634	24.464 ± 9.209	18.999 ± 4.017	0.203
M	26	0.644	18.541 ± 4.733	15.964 ± 3.224	0.516
MXy	26	0.500	26.408 ± 5.618	21.310 ± 5.525	0.095 ·
CW_{thick} (μm)					
MXy	16	0.000	1.503 ± 0.229	1.056 ± 0.227	0.001 ***

Note: Size of cells was represented by the maximum cell length (MCL) and mean cell wall thickness (CW_{thick}). n refers to sample size and λ refers to Pagel's estimator of phylogenetic signal. Significance level: · , $p < 0.1$ and ***, $p < 0.001$. Abbreviation: bulliform cells (BC), bundle sheath (BS), epidermis (E), mesophyll (M), minor vein (MV), and metaxylem vessels (MXy).

Correlation of minor vascular bundle size with cell size and cell number in grass leaves

Generally, each minor vascular bundle is made from minor vein tissue surrounded by one or two layers of bundle sheath cells. Each minor vein consists of two tissue types involved in the plant transport system, the xylem and phloem. Thus, we further analysed whether minor vein enlargement in grass leaves arose from differences in xylem and/or phloem tissues.

Our PGLS analysis shows that both xylem and phloem areas are positively correlated with MV area with p-value < 0.001 (Figure 2.6 (B) and (D)). This suggests that larger minor veins have both bigger xylem and phloem tissue areas. We further tested the correlation between climate and the areas of xylem and phloem. However, there was no difference in either tissue area between temperate and tropical species (Table 2.2). Additionally, we further questioned whether there is a relationship between the number of cells and sizes of the xylem and phloem. Due to the limitation of cross-sectional image resolution, we could not identify the type of cells in each tissue. Thus, we generally counted all cells in each tissue as either xylem or phloem cells. The result from PGLS analysis shows a positive regression between the number of cells and tissue area in both xylem and phloem with p-value < 0.001 (Figure 2.6 (A) and (C)). It can therefore be concluded that the size of minor veins depends on the size and the number of cells of both the xylem and the phloem. It can be argued that larger minor veins contain larger xylem and phloem tissues, each composed of greater cell numbers.

We further asked whether the larger bundle sheath is related to a greater number or larger size of cells. The PGLS result shows that bundle sheath areas of C_4 grass lineages are positively related to their cell number with a p-value < 0.01 (Figure 2.6 (E)). This means that the larger the bundle sheath area, the greater the cell number, suggesting that BS tissue area is influenced by the number of cells it contains.

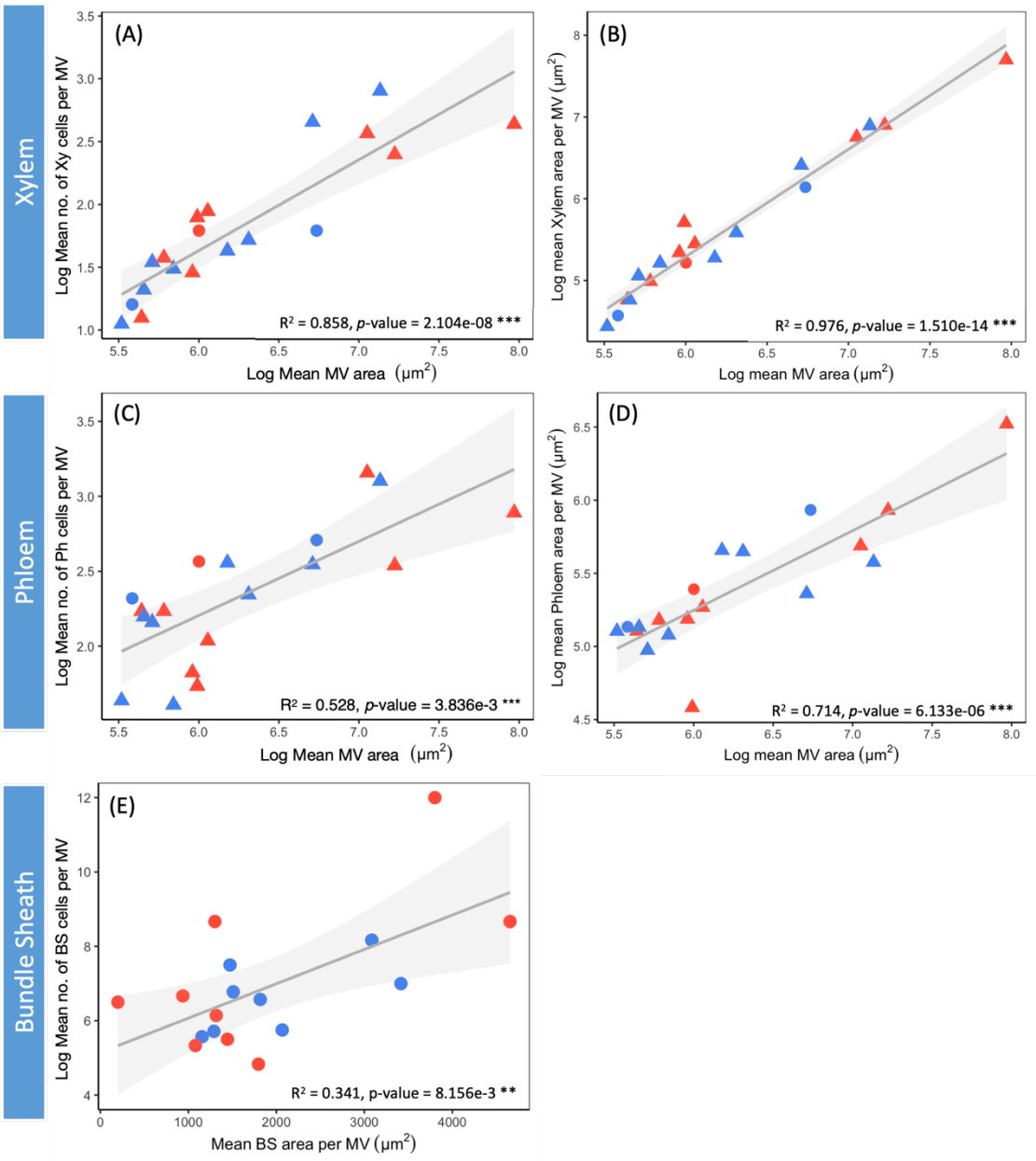

Figure 2.6 The relationship of minor vein and bundle sheath sizes, and the numbers of cells in different tissue types. Temperate and tropical species are shown in blue and red, respectively. The symbols represent photosynthetic types; ▲ is C_4 and ● is C_3. Abbreviation: bundle sheath (BS), minor vein (MV), xylem (Xy) and phloem (Ph). Significance level: **, $p < 0.01$; and ***, and $p < 0.001$.

Relationship between leaf anatomical traits and moisture content

According to a previous study (Watcharamongkol, 2019), low moisture content in leaves may promote C_4 grass tolerance to freezing. In this study, we tried to explain variation in leaf moisture content by testing for the relationship between the moisture content and the areas of distinct tissue types and the cell size of each particular tissue type.

Our PGLS analyses did not detect an effect of any of the measured tissue areas on leaf moisture content (Figure 2.7 and Table 2.3). To cope with the limitation of measuring leaf tissue areas, we focused on cell size instead by measuring the maximum cell length (MCL) of each tissue type. We used PGLS analysis to test whether the size of cells in any of the tissue types explains leaf moisture content. Our analyses show that among the different cell types, bulliform cells (BC), epidermis (E), mesophyll (M), and metaxylem (MXy) show a positive relationship with leaf moisture content (p-values = 0.022, 0.004, 0.0004, and 0.052, respectively; Table 2.3 and Figure 2.8). However, the adjusted R^2 of the models are relatively low (Table 2.3), which indicates that the cell size of those tissue types only explains a limited amount of the variation in leaf moisture content.

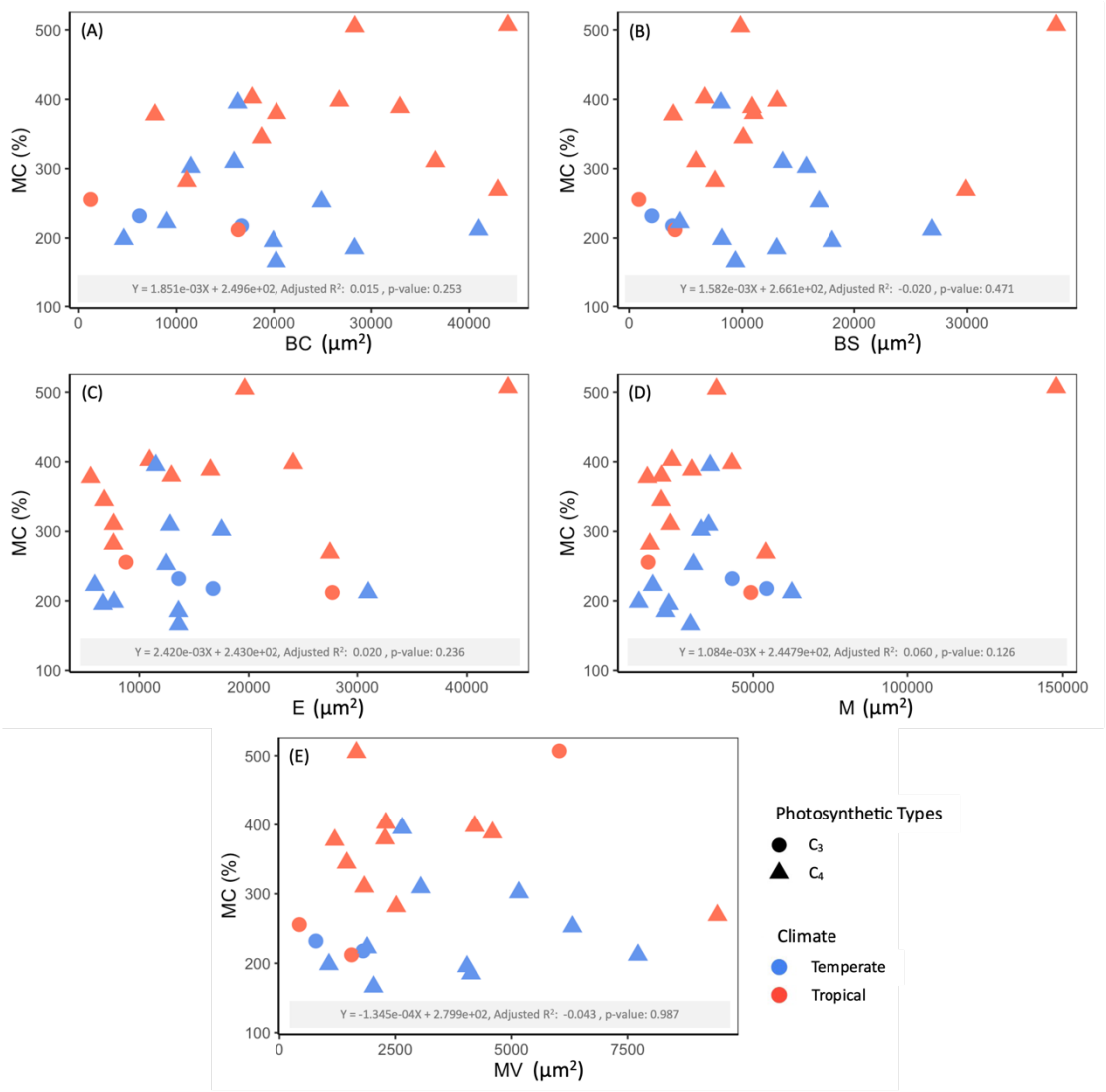

Figure 2.7 Relationship between the areas of different leaf tissues and leaf moisture content; (A) bulliform cells (BC), (B) bundle sheath (BS), (C) epidermis (E), (D) mesophyll (M), and (E) minor vein (MV). X-axis shows leaf moisture content (%) and y-axis shows the absolute area of each particular tissue type (µm^2).

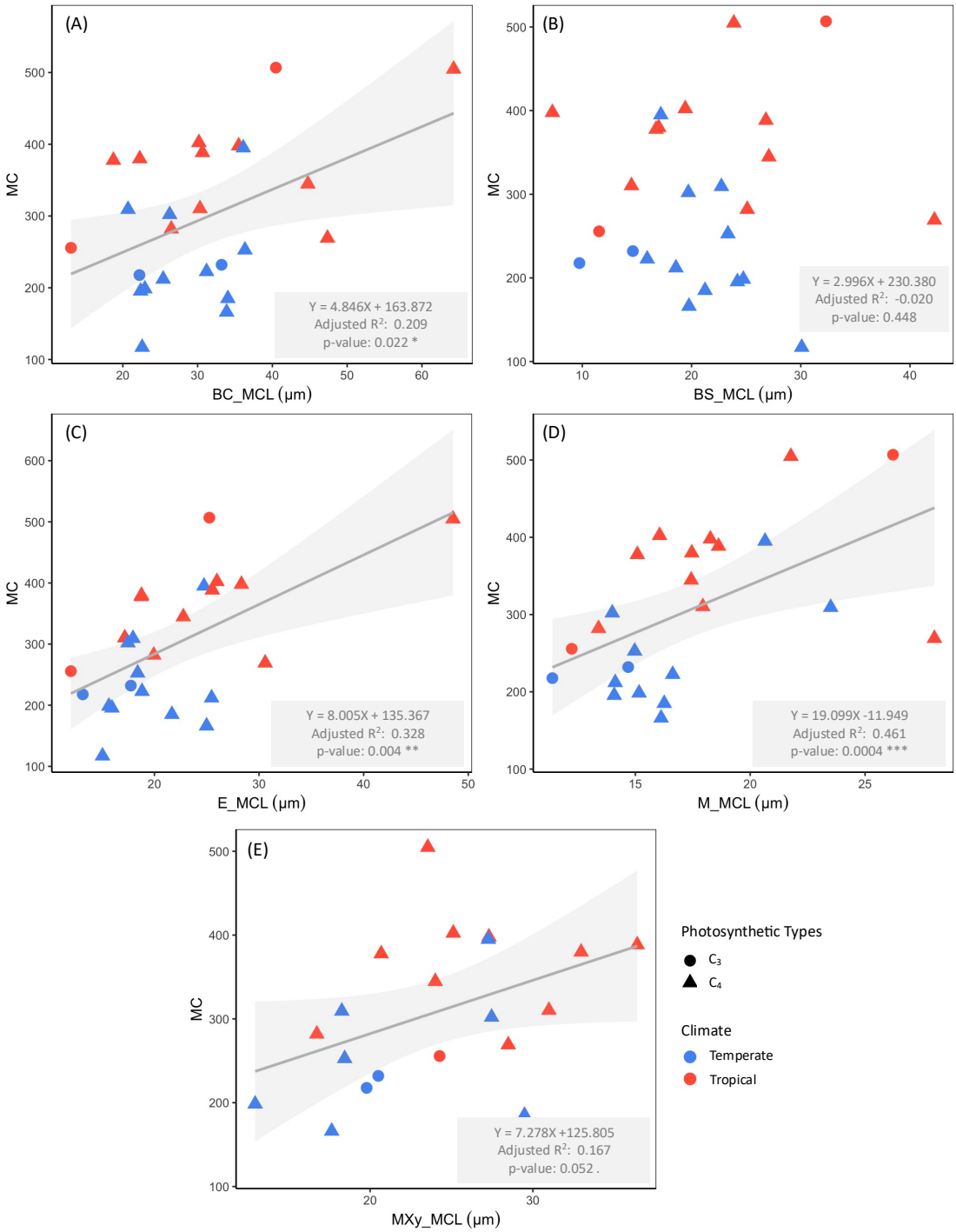

Figure 2.8 Relationship between the cell size of different leaf tissues and leaf moisture content; (A) bulliform cells (BC), (B) bundle sheath (BS), (C) epidermis (E), mesophyll (M), and (E) metaxylem vessels (MXy). X-axis shows leaf moisture content (MC) (%) and y-axis shows the maximum cell length (MCL). Significance level: . , $p < 0.1$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Table 2.3 Results of PGLS analysis testing for a relationship between both leaf tissue areas and cell size of each tissue type and leaf moisture content.

Variables	n	λ	Adjusted R^2	p-value
Tissue Area (μm²)				
BC	23	0.303	0.015	0.253
BS	23	0.359	-0.020	0.471
E	23	0.370	0.020	0.236
M	23	0.250	0.060	0.126
MV	23	0.454	-0.043	0.987
MCL (μm)				
BC	25	0.000	0.209	0.022 *
BS	25	0.542	-0.020	0.448
E	25	0.000	0.328	0.004 **
M	25	0.000	0.461	0.0004 ***
MXy	20	0.613	0.167	0.052 .

Note: Size of cells was represented by the maximum cell length (MCL) (μm). n refers to sample size and λ refers to Pagel's estimator of phylogenetic. Significance level: . , $p < 0.1$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Abbreviation; BC, bulliform cells; BS, bundle sheath; E, epidermis; FB, Fibre; M, mesophyll; MV, minor vein; and VB, vascular bundle.

Leaf anatomical adaptations to maintain photosynthetic efficiency at low temperatures; chilling and freezing temperatures.

(1) Chilling temperatures

To explain how grass leaf anatomy is related to the maintenance of photosynthetic efficiency at chilling temperatures, we used PGLS to examine the relationship between the cross-sectional leaf anatomy traits and the maximum quantum yield of photosystem II (PSII) after growing plants under chilling temperatures at day 1, 7, and 14. Our PGLS analysis shows no correlation of the maximum quantum yield of PSII after growing at a chilling temperature for 1 day (FvFmC1) and leaf anatomy traits (Table 2.4). However, after treating plants with chilling conditions for 7 days, the maximum quantum yield of PSII (FvFmC7) dropped in all species. We found only mesophyll (M) showed a negative correlation with a weak statistical significance (p-value = 0.079) (Figure 2.9 and Table 2.4). These results indicate that the smaller M area, the higher FvFmC7, suggesting that having a small M area is the key leaf adaptations to maintain photosynthetic efficiency at the low temperatures. Additionally, after chilling for 14 days, the result shows that there is no any relationship of any leaf tissue areas and the maximum quantum yield of PSII on day 14 (FvFmC14) (Figure 2.9 and Table 2.4). §

(2) Freezing temperatures

To explain how grass leaf anatomy is related to the maintenance of photosynthetic efficiency after a freezing event, we used PGLS analysis to test the relationship between leaf tissue area and the maximum quantum yield after freezing (FvFmC15). Our result obviously shows that MV show positive relationships with FvFmC15 with p-values of 0.067 (Figure 2.9 and Table 2.4). Thus, as with chilling temperatures, larger MV areas are associated with higher photosynthetic efficiency. Thus, this result suggests that to maintain photosynthesis at freezing temperatures, grasses need to evolve large MV area.

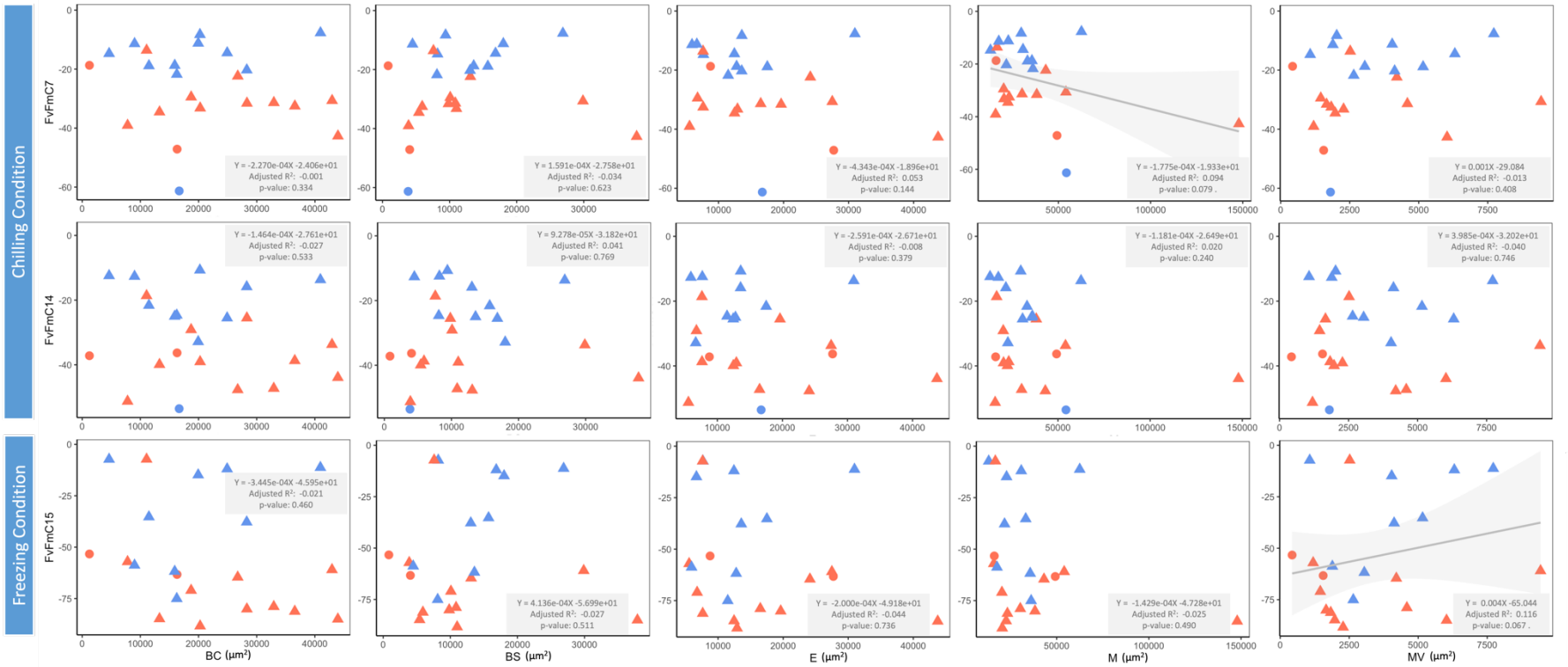

Figure 2.9 Relationship of tissue area and photosynthetic efficiency at chilling and freezing temperatures. Abbreviations: bulliform cells (BC), bundle sheath (BS), epidermis (E), mesophyll (M), and minor vein (MV). FvFmC7 and FvFmC14 are the maximum quantum yield of photosystem II (PSII) under chilling conditions at day 7th and 14th, respectively, while FvFmC15 is the maximum quantum yield of PSII under freezing conditions. Temperate and tropical species are shown in blue and red, respectively. Symbol is represented for photosynthetic types; ▲ is C_4 and ● is C_3. Significance level: · , $p < 0.1$.

Table 2.4 Results of PGLS analysis testing for a relationship between leaf tissue areas and photosynthetic efficiency of grasses after growing at chilling temperatures and followed by a freezing treatment.

	Variables	df	λ	Adjusted R^2	p-value
FvFmC1	BC	21	0.000	-0.005	0.356
	BS	21	0.000	0.012	0.272
	E	21	0.000	-0.045	0.832
	M	21	0.000	0.013	0.270
	MV	21	0.000	-0.002	0.341
FvFmC7	BC	22	0.239	-0.001	0.334
	BS	22	0.000	-0.034	0.623
	E	22	0.000	0.053	0.144
	M	22	0.000	0.094	0.079 .
	MV	22	0.000	-0.013	0.408
FvFmC14	BC	22	0.000	-0.027	0.533
	BS	22	0.000	0.041	0.769
	E	22	0.000	-0.008	0.379
	M	22	0.000	0.020	0.240
	MV	22	0.000	-0.040	0.746
FvFmC15	BC	20	0.399	-0.021	0.460
	BS	20	0.562	-0.027	0.511
	E	20	0.393	-0.044	0.736
	M	20	0.346	-0.025	0.490
	MV	20	1.000	0.116	0.067 .

Note: N refers to sample size and **λ** refers to Pagel's estimator of phylogenetic. Significance level: · , $p < 0.1$. Abbreviation; BC, bulliform cells; BS, bundle sheath; E, epidermis; M, mesophyll; and MV, minor vein. FvFmC1, FvFmC7, and FvFmC14 are the maximum quantum yield of photosystem II at day 1, 7, and 14 after chilling, respectively. FvFmC15 is the maximum quantum yield of photosystem II after freezing.

5. Discussion

Low temperature is one of the environmental factors that can affect plants, leading to changes in their physiological and biochemical processes (Ruelland et al., 2010). Alterations in leaf anatomy can significantly impact these processes and may therefore be used by plants to adapt to low-temperature conditions. Although leaf adaptation to low temperatures in grasses has been studied previously, the studies were limited to only certain species (Ahmad et al., 2016; Bilska-Kos et al., 2018). To broaden our understanding of how grass leaves have evolved to survive at low temperatures, it is necessary to compare leaf anatomy across multiple lineages.

In our first study, we compared the leaf tissue area and the cell size of each tissue type. Unexpectedly, our results show that the bundle sheath (BS) tissue area is not statistically different between tropical and temperate grasses. Furthermore, we did not find a statistical correlation between the BS area and the maximum quantum yield of photosystem II (Fv/Fm) at any time points under both chilling and freezing conditions. This lack of statistical significance may be due to the low number of biological replications, or the limited number of species used in this study. Our findings contradict those of Kubien and Sage (2004), who reported that RubisCo becomes the rate-limiting enzyme for C_4 photosynthesis under low temperatures, meaning that C_4 grasses may need to enlarge their RubisCo-containing cells (i.e., bundle sheath cells) to maintain their photosynthetic efficiency. However, our results align with Pignon *et al.* (2019), who found no evidence of enlarged BS chloroplast volume per leaf area in chilling-tolerant species relative to chilling-sensitive ones. This suggests that a large BS area is not necessarily related to RubisCo content in chloroplasts or directly influences photosynthetic efficiency at low temperatures. Additionally, Naidu and Long (2004), pointed out that RubisCo is not the only limitation for C_4 photosynthesis at chilling temperatures. Indeed, decreases in photosynthesis can have multiple causes such as damage to photosystem I or II (Kudoh et al., 2002; Wang et al., 2008) and a restriction of electron transport at low temperatures (Labate et al., 1989a). Moreover, comparative transcriptomics in *Miscanthus x giganteus* suggests that the chilling tolerance of photosynthesis relates to the up-regulation of genes encoding several photosynthetic proteins such as *lhcb5* (chlorophyll a/b-binding protein CP26), *ndhF*

(NADH dehydrogenase F, chloroplast), *atpA* (ATP synthase alpha subunit), *psbA* (D1), *petA* (cytochrome f), and *lhcb4* (chlorophyll a/b-binding protein CP29) (Spence et al., 2014). These studies indicate that various factors may influence BS size. Thus, focusing solely on RubisCo content as the determinant of BS cell size is insufficient. To gain a comprehensive understanding of BS adaptation to low temperatures in grasses, future studies should increase the sample size and species diversity and further explore the factors influencing BS cell size.

Minor veins (MVs) contain tissues that are involved in transport in plants, including xylem for water transport and phloem for sugar translocation (Schulz et al., 2009; Sack et al., 2013). Similar to the BS area, our studies show that there is no statistically significant difference between tropical and temperate species. However, we found a positive relationship between MV area and photosynthetic efficiency on the first day after freezing (FvFmC15), though not during chilling. This suggests that MVs may play a crucial role in preventing photosynthesis damage at freezing temperatures. Moreover, our results show that MV area is positively correlated to both xylem and phloem areas and the areas of both transport tissues are positively related to their cell number. These data indicate that grass leaves may evolve larger MV areas comprising larger vascular tissues and greater numbers of cells to maintain vascular transport and leaf photosynthesis at freezing temperatures. However, the relationship between MV area and FvFmC15 has low statistical power ($0.05 < \text{p-value} < 0.1$). To confirm this finding, future studies should increase the number of biological replicates and the diversity of species examined.

Metaxylem is the largest xylem vessel type located in major veins and midrib, which plays major roles in water transport and maintaining stomatal conductance at low temperatures (Sack et al., 2013). Our results show that temperate species have smaller metaxylem vessels with thinner cell walls than their tropical counterparts. This result is consistent with several findings that plants experiencing sub-zero temperatures such as grasses and trees living in temperate, alpine, and high altitudes have reduced metaxylem vessel size (Davis et al., 1999; Pittermann et al., 2006; Ahmad et al., 2016; Tamang et al., 2022). When plants experience freeze-thaw events, the co-occurrence of cellular ice formation and the decrease in xylem water pressure result in the formation of bubbles (or embolisms) in the xylem vessels. When the ice thaws, the air bubbles

may not be able to reabsorb into the water column due to surface tension and adhesion forces between water molecules and the xylem walls, resulting in the trapping of air bubbles and blocking the flow of water. To repair embolisms, plants need to increase xylem pressure over a water vapour bubble in the vessel via water refilling mechanisms, which can be both passive and active (Clearwater et al., 2005). Narrower vessels need less volume of water and time to refill comparing to the larger one (Vesala et al., 2003). Thus, having small metaxylem is crucial in helping to repair freeze-thaw embolism. However, the refilling mechanism requires nearby living cells and significant metabolic activity (Griffiths et al., 2013), Thus, the area-to-volume ratio of xylem- parenchyma contact may need to be considered. In addition, Brodribb and Feild (2000) show that photosynthetic electron transport is positively correlated to stem hydraulic conductivity in conifer and vesselless angiosperms. This suggests that plants need to maintain their water transport to maintain photosynthesis. Thus, to adapt to the low temperatures, having small metaxylem is needed for plants to maintain water transport leading to the maintenance of photosynthetic efficiency.

According to Watcharamongkol (2019), leaf moisture content is lower in temperate than tropical species. Thus, we further tested whether which leaf tissue(s) is/are related to the moisture content. Our phylogenetic analyses show there is no relationship between tissue areas and moisture content. However, there is a positive correlation of the sizes of mesophyll, epidermis, and bulliform cells with the leaf moisture content. This result is consistent with a previous report by Garnier and Laurent (1994) which stated that the proportion of mesophyll protoplast (i.e. the proportion of mesophyll minus proportion of mesophyll occupied by cell walls) is the best explanation for interspecific differences in leaf water content. Moreover, bulliform cells are enlarged, colourless epidermal cells and have a function involved in leaf rolling and expansion by regulating water intake (Metcalfe, 1960; Ellis, 1976). These cells may therefore play a crucial role and directly relate to leaf moisture content. Additionally, as bulliform cells are a part of the epidermis, this leads to epidermis cell size having a positive correlation with leaf moisture content also.

6. Conclusions

In this study, we investigated how C_4 grasses adapted to low temperatures, focusing on leaf anatomical changes among 26 grass lineages from two distinct climatic regions, temperate and tropical. We compared anatomical traits between 12 temperate and 14 tropical species. Our results show that temperate lineages tend to have smaller mesophyll (M) tissue areas than their tropical counterparts. According to Watcharamongkol (2019), temperate grasses have lower leaf moisture content than their tropical counterparts. Our results show that there are positive relationships between the moisture content and bulliform cells (BC), epidermis (E), mesophyll (M), and metaxylem (Mxy) cell sizes. Moreover, M is negatively related to photosynthetic efficiency after 7 days of chilling treatment. These findings suggest that temperate lineages tend to have a small M area to reduce the leaf moisture content, which is related to the prevention of ice crystal formation in cells or intercellular spaces, enabling the maintenance of photosynthetic efficiency at low temperatures. Furthermore, our results indicate that small Mxy in major veins is a key adaptation to low temperatures, potentially aiding in embolism repair and allowing continued water transport and photosynthesis under cold conditions. In conclusion, grasses have evolved leaf adaptations to low temperatures that are consistent across lineages, and these adaptations may be related to several physiological processes such as photosynthesis, water transportation, and translocation.

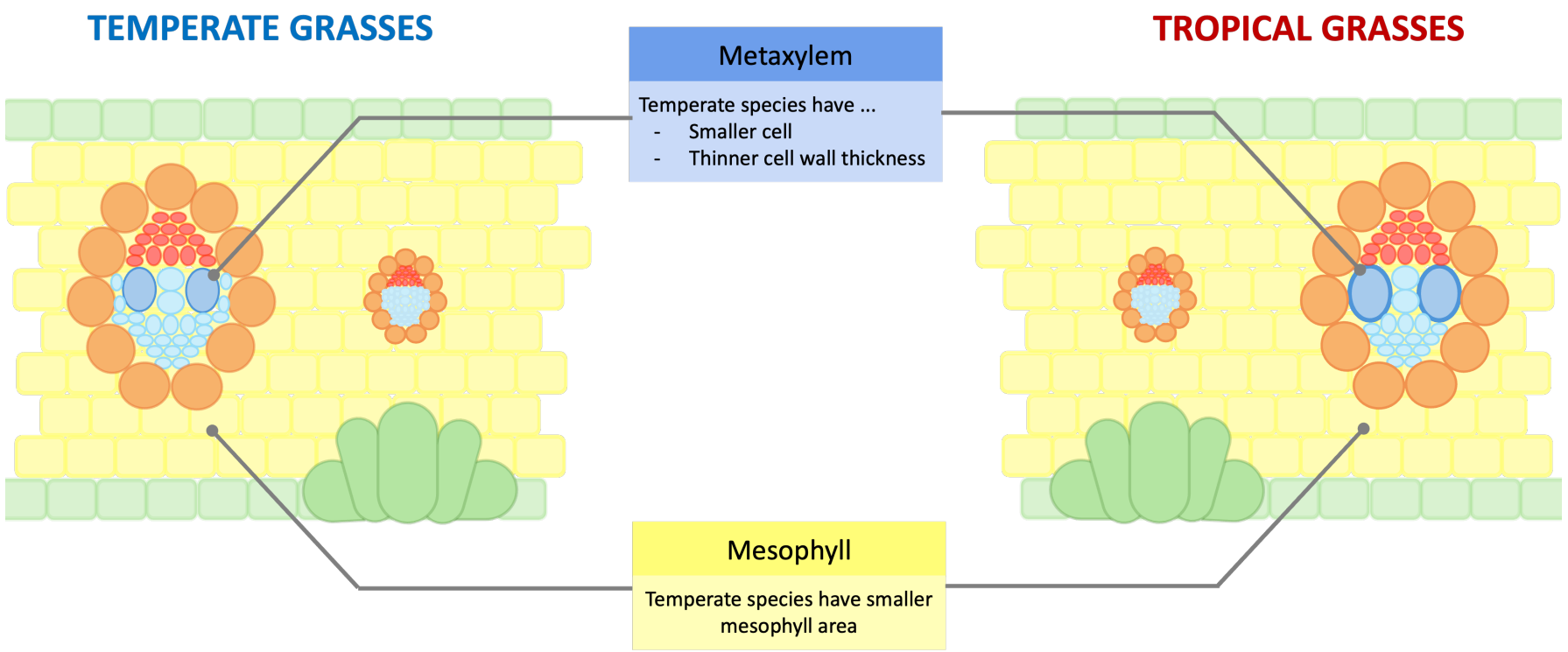

Figure 2.10 Interspecific leaf anatomical adaptations to low temperatures in grasses. Each colour corresponds to a distinct tissue type: light green represents epidermis (E), dark green represents bulliform cells (BC), and yellow denotes mesophyll (M). Orange indicates bundle sheath (BS), while red represents phloem (Ph). Blue denotes xylem (Xy), with large blue circles in major veins representing metaxylem vessels.

7. Acknowledgements

I would like to thank Alice Coppock, a former MRes student, for preparing leaf samples in resin blocks and producing slides of leaf cross-sections. Thanks to Dr Teera Watcharamongkol, a former PhD student for the datasets of cold and freezing tolerance of some C_3 and C_4 grasses. This work was funded by a studentship awarded by the Royal Thai Government.

Chapter 3

Intraspecific adaptations of leaf structures to low temperatures in *Alloteropsis semialata*

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Personal contribution:

I co-designed the study with Prof. Colin P. Osborne. I used leaf samples and microscope pictures prepared by Dr Matheus E. Bianconi and Vanja Milenkovic. I used image-processing software, ImageJ, to extract quantitative traits from the microscope pictures. I performed all analyses and interpreted the results. Finally, I wrote the paper with the help of Prof. Colin P. Osborne.

1. Abstract

Alloteropsis semialata is a grass species that exhibits a range of photosynthetic types including C_4, C_3-C_4 intermediate, and C_3, which have diverse geographic origins. This means that some *A. semialata* genotypes have developed adaptations to grow at low temperatures. Following my previous work on leaf adaptation to low temperatures (Chapter 2) that took a macroevolutionary approach by comparing different grass lineages, this study aims to elucidate how grass leaves within a single species adapt to low temperatures. To examine this intraspecific adaptation, I compared cross-sections for the leaves of 15 *A. semialata* genotypes from diverse geographic origins, which had been grown under a controlled environmental greenhouse with day/night temperatures of 25/20 ºC. Based on the findings from my previous study (Chapter 2), I expected that genotypes from cooler climate regions would develop a small metaxylem and larger minor vein and bundle sheath areas to compensate for decreased photosynthetic and transport efficiencies under low temperatures. Therefore, in this study, metaxylem size, minor vein (MV), bundle sheath (BS) areas, and the number of cells in those tissues were measured. The results reveal that MV areas in genotypes from colder regions were about four times larger than those from warmer regions. The MV area is associated with both a greater phloem tissue area and larger number of conduits within this phloem tissue. This result implies that the number of phloem elements increases to compensate for a slowing of phloem transport rate at low temperatures. Surprisingly, the BS area did not differ significantly among genotypes. However, the number of BS cells increased in genotypes from colder regions, which corresponded with the increase in MV area. Moreover, no differences in metaxylem size were observed between genotypes. Overall, this study underlines the consistency of leaf adaptations to low temperatures within a single grass species and shows some common adaptations across different lineages.

2. Introduction

Low temperatures are a major limiting factor for the biochemistry and physiology of plants (Metcalfe, 1960; Janská et al., 2010; Rihan et al., 2017; Szymańska et al., 2017; Ritonga et al., 2020; Bhattacharya, 2022). At low temperatures, chemical processes and gene expression are slowed down, leading to a decrease in the efficiency in various physiological processes such as photosynthesis (Hendrickson et al., 2004), respiration (ap Rees et al., 1988; Labate et al., 1989a), water transport (Sucoff, 1969), and translocation (Weatherley et al., 1969; Webb, 1971; Farrar, 1988). As a result, low temperature is the primary limiting factor for plant performance, growth and development (Wingler et al., 2016), as well as geographical distribution on a global scale (Woodward, 1988, 1990).

Grasses are a major plant group that has successfully migrated into areas characterised by low temperatures (Hopkins et al., 2006; Linder et al., 2018). Numerous species can grow and survive in the temperate and alpine regions. For instance, perennial ryegrass (*Lolium perenne* L.), creeping bentgrass (*Agrostis stolonifera L.),* and annual bluegrass (*Poa annua L.)* are found widely in temperate areas (Hoffman et al., 2014; Kovi et al., 2015), while alpine meadow-grass (*Poa alpina*), blue wild rye (*Leymus secalinus*), and Junegrasses (*Koeleria pers*) are found in high altitude regions (Abalori et al., 2022). Despite their origins in tropical and warm temperate climates (Watcharamongkol et al., 2018), there are several genotypes of C_4 crop grasses that are cultivated at low temperatures such as maize (*Zea mays*) and Mammoth Miscanthus (*Miscanthus x giganteus*) (Wang et al., 2008; Farooq et al., 2009; Głowacka et al., 2014; Yin et al., 2016).

To survive under low temperatures, plants that can adapt or develop suitable traits are better equipped to survive and migrate to cold areas. Several studies have shown that grasses have developed characteristics to maintain and compensate for metabolic and physiological processes at low temperatures. For example, *M. x giganteus* can partially recover light-saturated CO_2 assimilation (A_{sat}) and quantum yield of photosystem II (Φ_{PSII}) within 48 hours after it is transferred from 25 to 14 °C and maintain these parameters after that (Wang et al., 2008). This physiological acclimation response can be explained by increases in photosynthetic rate-limiting

enzymes, ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCo) and pyruvate inorganic phosphate dikinase (PPDK) (Wang et al., 2008; Long et al., 2013), and the enlargement of bundle sheath cells, where is RubisCo found in C_4 plants such as a chilling-tolerant *Zea mays* (Bilska-Kos et al., 2018). A further risk to plants under low temperatures is posed by freeze-thaw events, the ice forming when the temperature reaches a freezing point trapping air bubbles in the ice crystal. Then when the ice thaws, the bubbles do not dissolve causing cavitation (also known as embolism) in xylem vessels, disrupting water transport. To cope with this problem, Scutch grass (*Cynodon dactylon*), a C_4 grass living at high altitudes, reduces the lumen area of metaxylem vessels (Ahmad et al., 2016) to increase the hydraulic conductivity of xylem, resulting in the maintenance of water transport function under low temperatures (Choat et al., 2011). Moreover, for sugar loading in phloem, chilling-tolerant maize has a less frequent plasmodesmatal network inside the phloem than chilling-sensitive maize, suggesting that the chilling-tolerant variety uses apoplastic phloem loading more than symplastic loading pathway (Sowiński et al., 2001). Although this work describes how phloem anatomy adapts to low temperatures, there are few studies in grass lineages and more studies are needed to explain the underlying mechanisms.

In our previous study (Chapter 2), we studied interspecific leaf adaptations to low temperatures by comparing temperate and tropical representatives of different C_4 grass lineages. Our results show that C_4 grasses originating from cold or temperate climatic regions tend to have larger minor vein and bundle sheath areas in comparison with their tropical relatives. We interpret these traits as compensating for the depression in photosynthetic and transport efficiencies at low temperatures. Moreover, we found that temperate grasses have smaller metaxylem vessels than their tropical relatives, which is expected to reduce freeze-thaw embolism in the vessels maintaining water transport for plant growth and photosynthesis. From this study, it can be concluded that the evolution of leaf anatomical adaptations to low temperatures shows consistency among C_4 grass lineages. To gain a better understanding of how leaf anatomical adaptations to low temperatures evolve, we want to further explain whether these particular adaptations are necessary for grasses to survive in cold climates and therefore evolved immediately or whether they have developed later to optimise physiological

processes in cool conditions. To address these questions, we have selected *Alloteropsis semialata* as a plant model.

A. semialata is a grass species that has variations in photosynthetic processes, including C_4, C_3-C_4 intermediate, and C_3 types (Lundgren et al., 2016). Additionally, the difference in these genotypes is found across geographical regions, which have a range of mean annual temperature (MAT) between 15 and 25 °C and mean annual precipitation (MAP) between 500 and 2000 mm (Fick et al., 2017). These special characteristics of this species make it a model for studying the evolution of adaptations to low temperatures in grasses. Although it is known that *A. semialata* lives and survives across a broad temperature range, the study of the underlying adaptations to low temperatures, especially leaf structural adaptations, is still limited.

To elucidate the intraspecific adaptations of leaf structure to low temperatures, we compared leaf transverse sections of 15 different *A. semialata* genotypes originating from different geographic origins. Based on the results from our previous study (Chapter 2), we focused our work on the areas of the bundle sheath, minor vein, phloem and xylem, and the size of metaxylem vessels. We hypothesised that intraspecific adaptations will be the same as interspecific ones. Our study shows that there are some commonalities in the leaf structural changes arising from interspecific adaptations among C_4 grass lineages and intraspecific adaptations in *A. semialata*. However, our work particularly highlights the importance of leaf structural adaptations for maintaining phloem transport at low temperatures.

3. Materials and methods

Plant material and environmental conditions

15 genotypes of *A. semialata* from different geographic origins were used in this study (Supplementary: Table S3.1). Briefly, the plants were cultivated in pots using a mixture of M3 compost (Levington) and perlite (Sinclair) in a ratio of 2:1 and kept well-watered. They were fertilised once every three months using an NPK fertiliser 16-0-5 with iron (Evergreen Extreme Green Lawn Food). The plants were grown in a controlled-environment greenhouse with a 12-hour day/night cycle, supplemented with metal halide lamps providing an additional 200 μmol m^{-2} s^{-1} at bench level, and maintained at a day/night temperature of 25/20 °C with relative humidity ranging from 30% to 60% and the ambient CO_2 concentration (Bianconi et al., 2021).

Investigation into the anatomical and structural changes in *A. semialata* leaves

Leaf cross-sections were prepared from the plant materials grown in the greenhouse (mentioned in the previous section) by Dr Matheus E. Bianconi and Vanja Milenkovic, our previous lab members. The third leaf from the top was collected from each particular genotype. One cross-sectional slide was prepared from the middle of the leaf including either side of the midrib. Detailed information on the preparation of leaf cross-sections was provided by Bianconi et al. (2021). Briefly, fresh leaf samples were dehydrated in an ethanol series from 70% to 100% ethanol and resin embedded with Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany) following the manufacturer's instructions. The embedded leaves were cut by using a microtome (Leica RM 2245, Leica Biosystems Nussloch GmbH, Nussloch, Germany), and stained with Toluidine Blue O (Sigma-Aldrich, St. Louis, MO, USA). Finally, images of cross-sections were taken with an Olympus DP71 camera (Olympus Corporation, Tokyo, Japan) before using an image-processing program, ImageJ (Schneider et al., 2012), to measure leaf anatomy traits.

Major veins or secondary veins are the veins that are ribbed with sclerenchyma (Esau, 1977) and contain metaxylem, the largest vessels in xylem (Figure 3.1 (A) and (B)). All metaxylem vessels located in the major veins of a leaf cross-section of each genotype were first identified. We measured the maximum cell length (MCL) and maximum lumen length (MLL) of every vessels in a particular leaf cross-section (Figure 3.1 (C)) and then calculated the means for each particular genotype. Mean cell wall thickness (CW_{thick}) was then calculated from the Equation 3.1.

$$CW_{thick} = \frac{MCL-MLL}{2} \qquad \text{Equation 3.1}$$

To measure minor vein, phloem, xylem, and bundle sheath areas, we focused on only one area that is located between the second and third secondary veins counted from a mid-vein. All veins that are located between these secondary veins were classified as minor veins (equivalent to intermediate veins) (Figure 3.1 (A) and (B)). ImageJ (Schneider et al., 2012) was used in the area determination. The number of cells of each tissue was counted for each particular minor vascular bundle and means were calculated for each genotype to use for further statistical analysis.

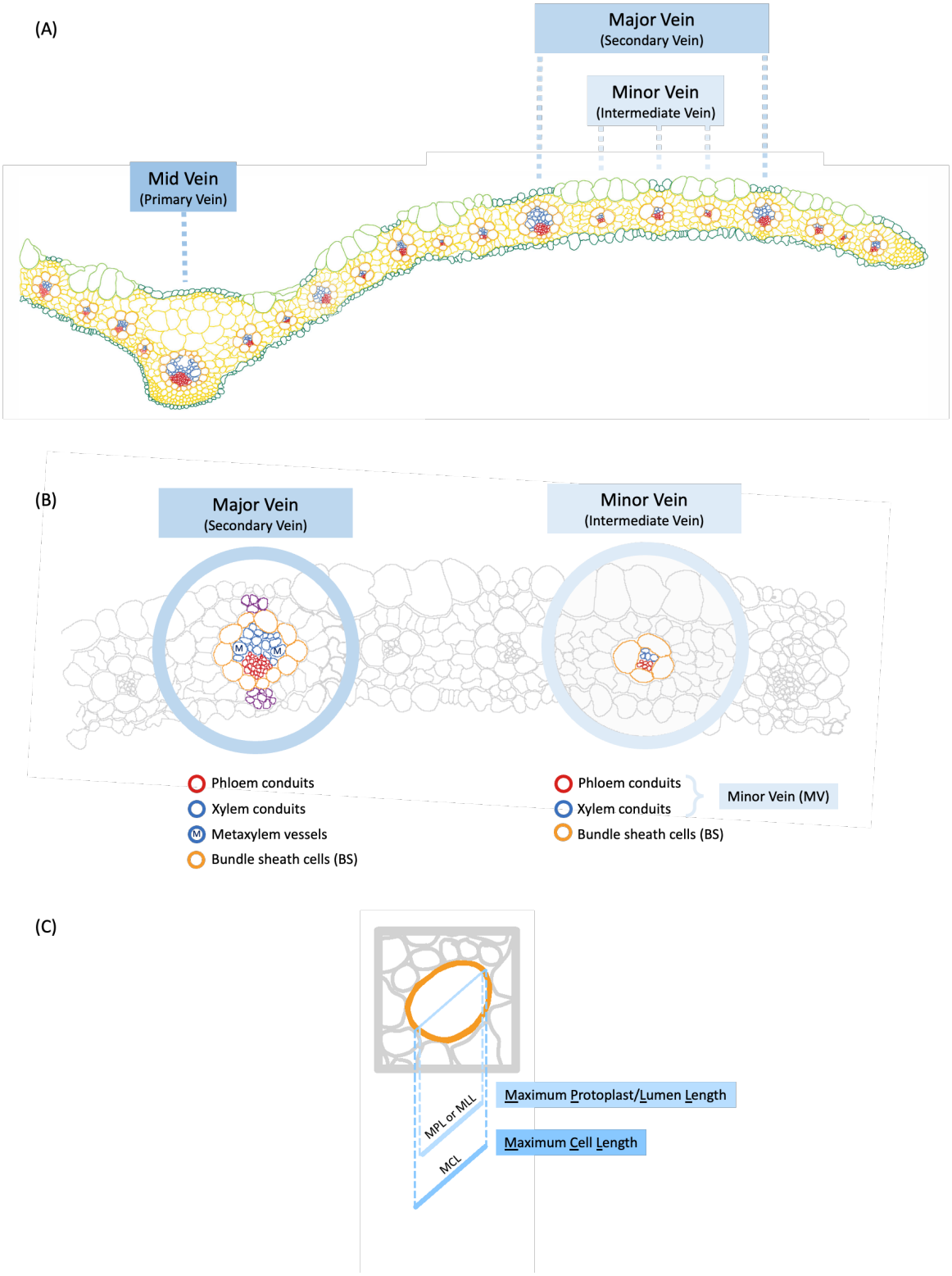

Figure 3.1 Leaf cross-section; (A) the positions of different kinds of veins, (B) the differences between major and minor veins and (C) metaxylem vessel measurement.

Bioclimatic dataset

Bioclimatic data for each genotype was downloaded from the public dataset WorldClim (Fick et al., 2017) by using global positioning system (GPS) data, latitude, and longitude, obtained during the collection of each genotype. The downloaded data were extracted by using in-house R code to obtain 19 bioclimatic variables (Supplementary: Table S3.2). These bioclimatic variables are not independent and are correlated with one another. Thus, we used a principal component analysis (PCA) using the function *prcomp* in R version 4.2.2 performed on these 19 bioclimatic variables to reduce the number of these variables into the principal components (PCs) describing the total variation in climate.

Statistical analyses

Phylogenetic generalised least squares (PGLS) was carried out using the package *caper* in R version 4.2.2 to test for relationships of climate and the size and number of cells in each tissue type, while accounting for the phylogenetic relationships among genotypes. The PC1 and PC2 from the PCA analysis, as well as mean annual temperature (MAT) and mean annual precipitation (MAP) were the independent variables, with photosynthetic types as the categorical predictor. Phylogeny was incorporated to control for phylogenetic dependence and to estimate the a's λ (Pagel, 1999; Freckleton et al., 2002). The phylogenetic tree of *A. semialata* used in this analysis is a time-calibrated phylogenetic tree based on nuclear genomes which is explained in Raimondeau et al. (2022) .

4. Results

Minor vein and phloem areas are correlated with migration to cold areas in *A. semialata.*

Our results revealed a negative correlation between mean minor vein area and mean annual temperature (MAT), as illustrated in Figure 3.2 (A). This indicates that the genotypes from colder areas have evolved to have larger minor vein areas, which is consistent with our findings in Chapter 2, where we observed that temperate C_4 grass lineages have larger minor vein areas than their tropical counterparts. However, we did not observe any relationship between minor vein area and mean annual precipitation (MAP) (Figure 3.2 (B)), nor with the first two principal components (PC) of the climate data obtained from the PCA analysis (Supplementary: Figure S3.2). These results suggest that precipitation may not be a significant factor in the evolution of minor veins in *A. semialata*, or that its influence may be negligible.

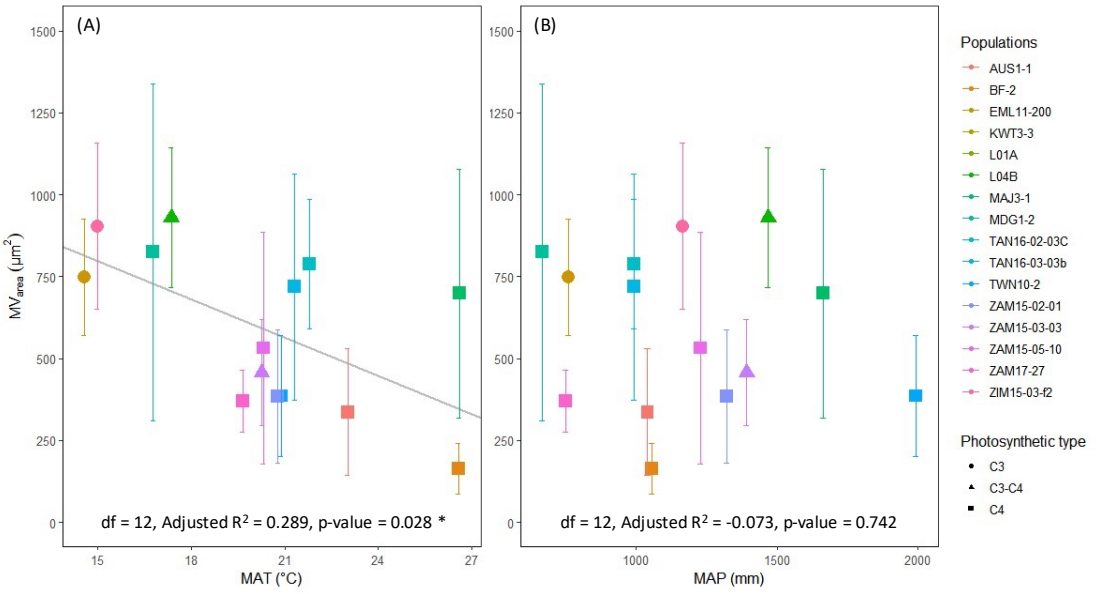

Figure 3.2 Relationships between the area of each minor vein for 15 genotypes of *A. semialata* from different geographic origins and two climate factors: (A) mean annual temperature (MAT) and (B) mean annual precipitation (MAP). Significance level: *, $p < 0.05$.

We further asked whether vascular tissues, phloem and/or xylem, are associated with a minor vein area and whether they are correlated with the migration of *A. semialata* to cold areas. Our results show that phloem areas have a negative correlation with MAT (Figure 3.3 (C)) but no relation with MAP (Figure 3.3 (D)), whereas there is no climate effect on xylem areas (Figure 3.3 (A) and (B)).

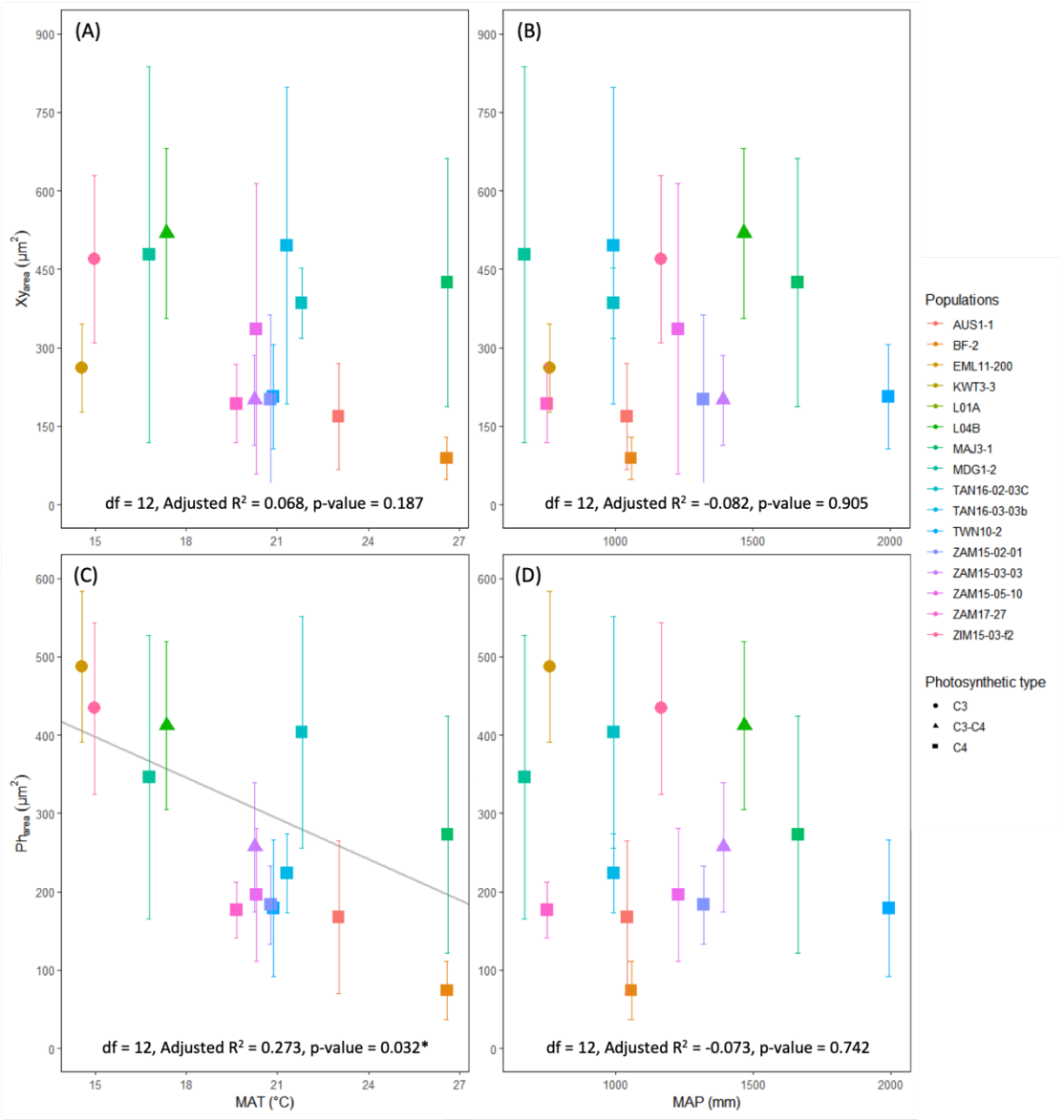

Figure 3.3 Relationships of vascular tissue areas, xylem (top) and phloem (bottom), climate variables, mean annual temperature (MAT) (left) and mean annual precipitation (MAP) (right). Significance level: *, $p < 0.05$.

Finally, our analysis reveals a strong relationship between the size of minor veins and phloem areas (Figure 3.4 (A)). These findings support the hypothesis that the increase in minor vein size is due to the increase in phloem rather than xylem tissue area, which may be a key adaptation mechanism in *A. semialata* for migrating to cold regions. Additionally, we observed a positive correlation between the size of minor vein phloem and its cell count (Figure 3.4 (B)). Specifically, the number of phloem cells increases with the increase in phloem area, with a coefficient value of 0.803, an adjusted R^2 of 0.848, and a p-value of 1.855e-0.6. This coefficient is significantly lower than 1.0, showing that phloem cell numbers do not scale isometrically with phloem area, and meaning that cell size is slightly enlarged overall when the phloem tissue area is larger.

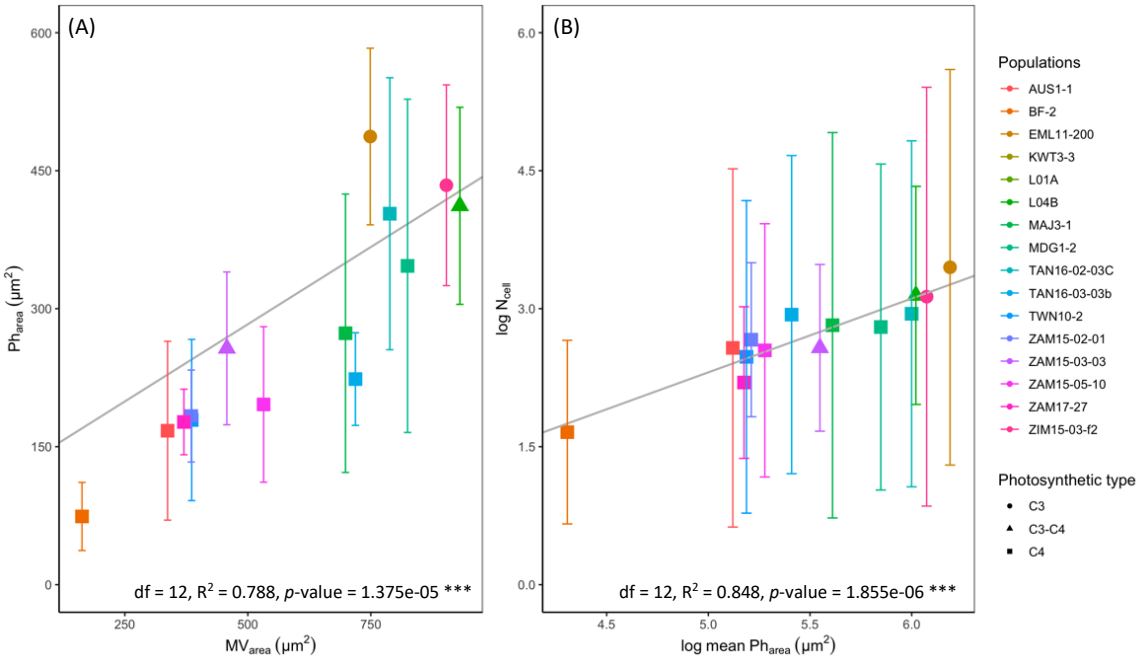

Figure 3.4 Relationships of phloem area and (A) minor vein area and (B) the number of phloem cells. Significance level: ***, $p < 0.001$.

Based on Figure 3.4, mean phloem area is the total area of phloem per minor vein (Ph_{area}). As we know the number of phloem cells per minor vein (N), we can calculate the average area of each particular phloem cell (Ph_{cell_area}) by using Equation (3.2).

$$Ph_{area} = N \cdot Ph_{cell\_area}$$

$$\text{then} \quad Ph_{cell\_area} = \frac{Ph_{area}}{N} \qquad \text{Equation (3.2)}$$

If we assume that the cross-sectional shape of each particular phloem cell is circular, then the radius of each cell (Ph_{cell_radius}) can be calculated by Equation (3.3)

$$Ph_{cell\_area} = \pi \cdot (Ph_{cell\_radius})^2$$

$$\text{then} \quad Ph_{cell\_radius} = \left| \sqrt{\frac{Ph_{cell\_area}}{\pi}} \right| \qquad \text{Equation (3.3)}$$

We have further tested the climatic effect on estimates of phloem cell area and its radius. Our PGLS result shows that TAN16-02-03C and MDG1-2 show the highest phloem cell area about 21 µm^2 and phloem cell radius about 2.6 µm, whereas TAN16-03-03b have the lowest phloem cell area and radius, which are 11.9 µm^2 and 1.9 µm, respectively. However, there is no systematic variation in cell area and radius in relation to climate among 15 *A. semialata* genotypes (Figure 3.5 and Table 3.1).

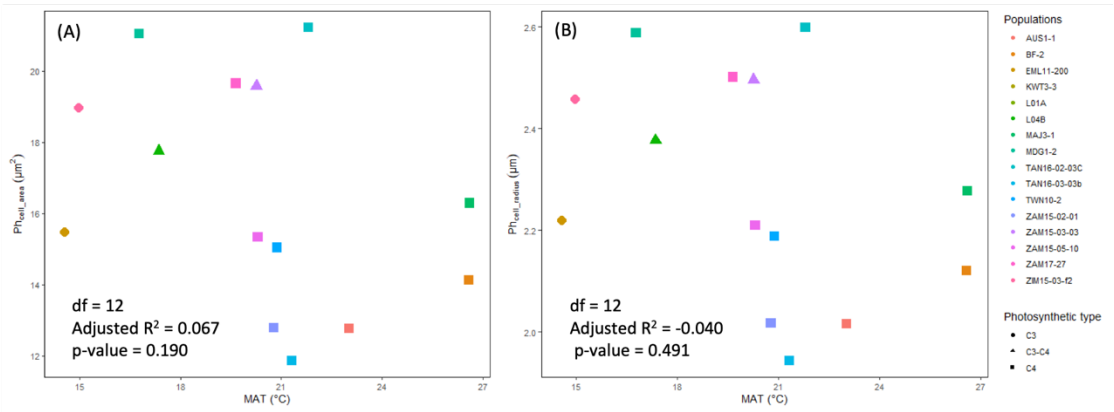

Figure 3.5 Relationship of phloem anatomical traits and mean annual temperature (MAT); (A) the average area of each phloem cell (Ph_{cell_area}) and (B) the radius of each cell (Ph_{cell_radius}).

Table 3.1 Results of PGLS testing for the effect of climate factors on the bundle sheath and minor vein traits.

Climate factors	Variables	df	λ	Estimate	Adjusted R^2	p-value
MAT	**Tissue Area** (μm^2)					
	MV_{area}	12	0.000	1379.719	0.289	0.028 *
	Xy_{area}	12	0.000	616.84	0.068	0.187
	Ph_{area}	12	0.602	657.930	0.273	0.032 *
	Phloem traits					
	Ph_{cell_area} (μm^2)	12	0.000	23.116	0.067	0.190
	Ph_{cell_radius} (μm)	12	0.000	2.427	-0.040	0.491
MAP	**Tissue Area** (μm^2)					
	MV_{area}	12	0.215	699.666	-0.073	0.742
	Xy_{area}	12	0.000	332.757	-0.082	0.905
	Ph_{area}	12	0.215	699.666	-0.073	0.742

Note: The climate factors are mean annual temperature (MAT) and mean annual precipitation (MAP). df refers to degree of freedom and λ refers to Pagel's estimator of phylogenetic. Significance level: · *, $p < 0.05$. Abbreviation; MV, minor vein; Xy, minor vein xylem; and Ph, minor vein phloem.

We further investigated whether the size of the phloem is related to the MV area. Our PGLS analysis results show no statistically significant relationships for both phloem cell area (Ph_{cell_area}) and phloem individual cell radius (Ph_{cell_radius}) with MV area (Figure 3.6 (A) and (B)). Interestingly, we found a strong positive relationship between the number of phloem cells and the minor vein area (p-value = 9.553e-05) (Figure 3.6 (C)). This indicates that the size of a minor vein is related to the number of phloem cells rather than their size; larger minor veins have a greater number of phloem cells.

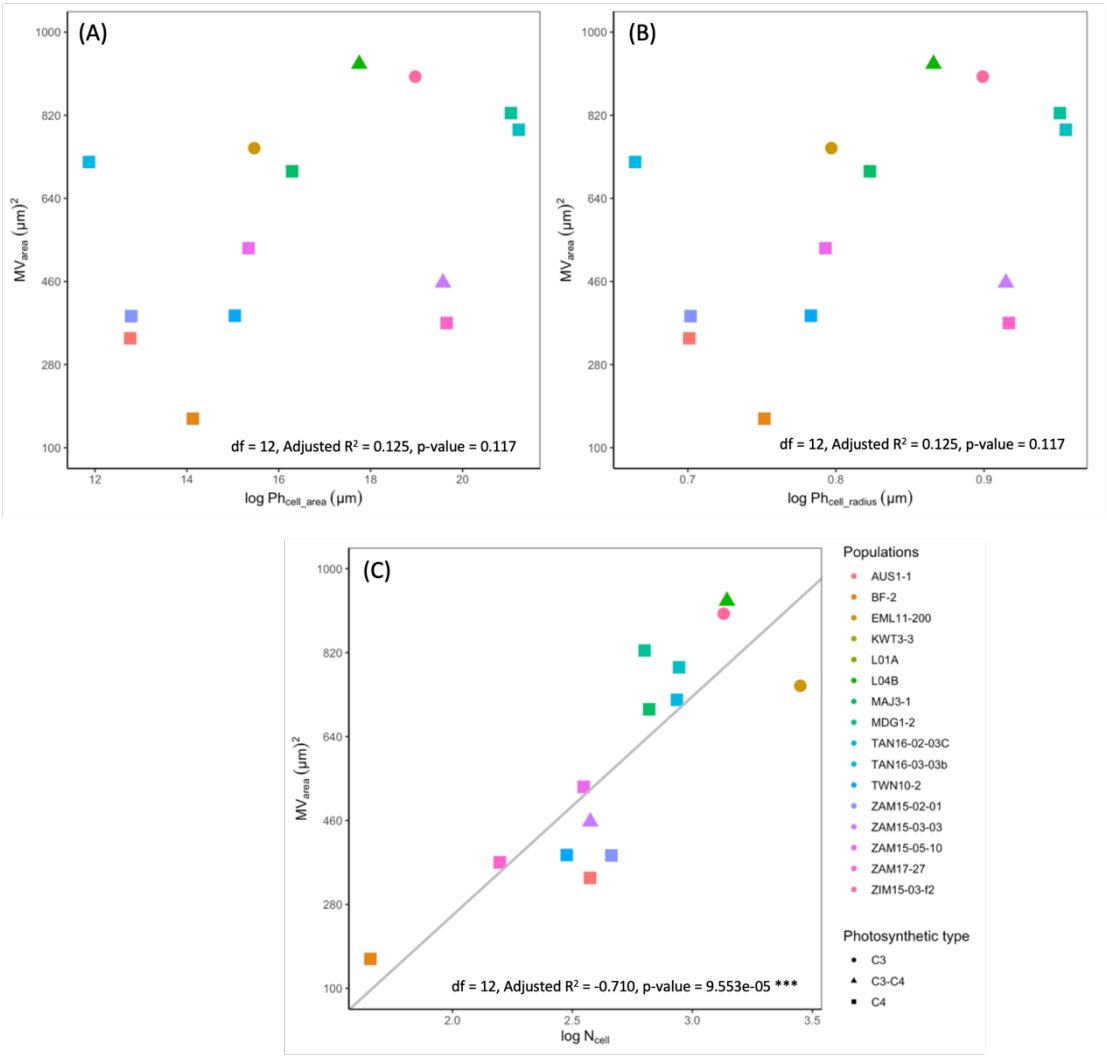

Figure 3.6 Relationship of minor vein area (MV_{area}) and phloem traits: (A) individual cell area (Ph_{cell_area}), (B) individual cell radius (Ph_{cell_radius}), and (C) number of phloem cells per a minor vein (N_{cell}). Significance level: ***, $p < 0.001$.

Greater numbers of larger phloem cells may help maintain phloem flow rate in *A. semialata* genotypes from colder areas

To help understand how a larger number of phloem cells may compensate for phloem transport, we applied a mathematical model of phloem flow rate based on previous studies in other plant species.

Based on Jensen et al. (2013), the flow of phloem sap through an individual phloem cell can be represented by Equation (3.4):

$$Q_C = \frac{\Delta p}{\eta} \cdot X \qquad \text{Equation (3.4)}$$

Where Q_C is the volumetric flow rate of phloem sap, Δp is the pressure differential driving the flow, η is the viscosity of the sugar solution, and X is a geometric parameter representing the shape and size of phloem cells. If phloem cells are represented as cylinders with radius r, and length L, then X become

$$X = \frac{\pi \cdot r^4}{8L} \qquad \text{Equation (3.5)}$$

If we want to represent the flow of phloem sap through a particular cross section of the leaf Q_L which have the total number of phloem cells within a cross-sectional area of the leaf N, it can be presented in Equation (3.6) and (3.7):

$$Q_L = \frac{\Delta p}{\eta} \cdot X \cdot N \qquad \text{Equation (3.6)}$$

or $$Q_L = \frac{\Delta p}{\eta} \cdot \frac{\pi \cdot r^4}{8L} \cdot N \qquad \text{Equation (3.7)}$$

According to Sowiński et al. (2001), chilling tolerant maize uses an apoplastic phloem loading pathway more than a symplastic pathway. Moreover, Jensen et al. (2013) show that several grass species (i.e. maize, barley, ryegrass, and wheat) use an apoplastic loading mechanism for phloem transport. Hence, in this study we assumed that *A. semialata* is an apoplastic phloem loader.

In apoplastic phloem loaders, we expect that Δp will be generated by the activity of sucrose transporters (SUTs), which are energised by a proton motive force created by plasma membrane H^+-ATPases (Williams et al., 2000; Sauer, 2007). Based on this assumption, Δp ultimately depends on ATPase activity. In sugar beet root, the plasma membrane ATPase activity drops from 100.7 to 23.9 nmol P_i (mg protein, min)$^{-1}$, which represents around a 75% reduction of activity, when the temperature falls from 25 to

15 °C (Figure 2, Lindberg et al., 2005). Following the argument outlined above, this decreased ATPase activity is expected to cause an equivalent drop in the phloem flow rate through a particular leaf area (Q_L) of 75%.

Generally, viscosity of sugar solutions (η) is also temperature-related, such that η is increased at low temperatures, leading to a slower phloem flow rate (Telis et al., 2007). Additionally, the average optimal sugar concentration in phloem sap for active loading species is 21.6% w/w (Jensen et al., 2013). Based on Table 1 in Telis et al. (2007), if temperature decreases by 10 °C from 25 to 15 °C, the viscosity of a 20% w/w of sucrose solution increases by about 25 % (from 1.70 to 2.26 mPa·S). Consequently, this increased viscosity leads to a reduction of phloem flow rate (Q_L) of about 25%.

In combination, the 75% decrease in the pressure differential driving the flow (Δp) and the 25% increase in viscosity (η) when the temperature drops about 10 °C, from 25 to 15 °C, would therefore be expected to slow phloem flow ($Q_{L,25\rightarrow15°C}$) to 20%. The calculation is shown below.

If $Q_{L,25\rightarrow15°C}$ is the phloem flow rate after the temperature falls from 25 to 15 °C and $Q_{L,25°C} = \frac{\Delta p}{\eta} \cdot X \cdot N$, then.

$$Q_{L,25\rightarrow15°C} = \frac{(100-75)\% \cdot \Delta p}{(100+25)\% \cdot \eta} \cdot X \cdot N$$

$$Q_{L,25\rightarrow15°C} = \frac{25\% \cdot \Delta p}{125\% \cdot \eta} \cdot X \cdot N$$

$$Q_{L,25\rightarrow15°C} = 0.20 \cdot \frac{\Delta p}{\eta} \cdot X \cdot N \qquad \text{Equation (3.8)}$$

Or $\quad Q_{L,25\rightarrow15°C} = 20\%\ Q_{L,25°C}$

Based on Equation (3.8) and our result that individual phloem cell area and radius do not systematically vary with MAT among the 15 *A. semialata* genotypes (Figure 3.5 and Table 3.1), the geometric parameter of phloem cells (X) is a constant in our model. Therefore, to compensate for the decrease in phloem flow rate due to decreased ATPase activity and increased sap viscosity, the total number of phloem cells (N) must be increased approximately five-fold. Our observations show that the N per minor vein increases about two-fold, from 10.60 to 22.78, when the temperature drops from 25 to 15 °C (Figure 3.4 (B), and Supplementary: Table S3.3). This finding suggests that the two-fold increase in N of among *A. semialata* may not be sufficient to compensate for the expected effects of temperature within each phloem cell.

Based on Equation (3.7), even a small increase in the radius of an individual phloem cell (r or Ph_{cell_radius}) leads to a large effect on the phloem flow rate. Although the calculated individual phloem cell radius does not significantly vary in relation to MAT among *A. semialata* genotypes (Supplementary: Table S3.3), the raw data show a range of values. In Figure 3.5, TAN16-02-03C has the highest phloem cell radius of 2.6 µm, whereas TAN16-03-03b has the lowest phloem cell radius of 1.9 µm. We used a sensitivity test to predict the maximum and minimum phloem flow rate when the temperature drops from 25 to 15 °C by using the raw maximum and minimum phloem radius. The result shows that the phloem flow rate would be increased about 3.5-fold when the radius increases from 1.9 to 2.6 µm.

Altogether, the combined effect of increase in phloem cell number and phloem cell radius, which can increase the phloem flow rate by two-fold and 3.5-fold, respectively, suggests that the flow rate in total could increase up to 7-fold, which would be more than sufficient to compensate for the loss of phloem flow rate due to low-temperature impacts. However, previous findings suggest that the increases in cell numbers and cell radius are not the only factors helping plants to compensate for slower phloem flow rates. Based on Venturas et al. (2017), there are other factors that need to be considered, such as the length of conduits (L) and other factors that increase the pressure differential driving the flow (Δp).

No climate effect on metaxylem vessel size and its cell wall thickness

In our previous study on interspecific leaf adaptations to low temperatures, we found that temperate C_4 grasses have evolved a smaller metaxylem lumen size and thinner metaxylem cell wall than their tropical relatives (Chapter 2). To investigate whether similar adaptations occur in *A. semialata*, we examined the correlation between metaxylem size and climate. However, our results did not show any significant relationship between metaxylem size (MLL) or cell wall thickness with either mean annual temperature (MAT) or mean annual precipitation (MAP) (Figure 3.7), nor with the principal component (PC) 1 and 2 of PCA analysis of climate data (Supplementary: Figure S3.3).

Additionally, previous studies have suggested that increasing ploidy size is associated with larger cell size in plants (Tsukaya, 2008; Katagiri et al., 2016). Therefore, we also investigated whether ploidy number influenced metaxylem size or not. However, we found no relationship between ploidy number and metaxylem size or its cell wall thickness (Supplementary: Figure S3.4).

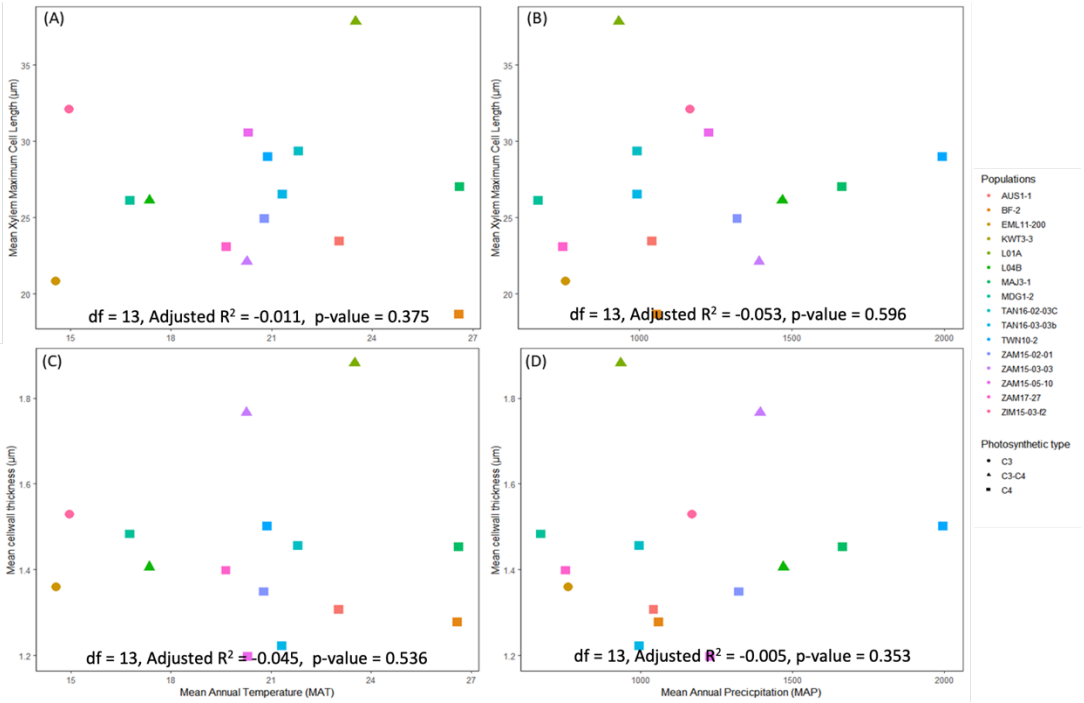

Figure 3.7 Relationship of metaxylem size and cell wall thickness and climatic factors, mean annual temperature (MAT) and mean annual precipitation (MAP).

Bundle sheath size is influenced by climatic factors but may be correlated with the size of minor veins

According to the study of leaf adaptations among different grass lineages in Chapter 2, the leaves of C_4 grass lineages with temperate origins have a larger bundle sheath (BS) area compared to their tropical counterparts. To examine how the BS adapted to low temperatures in *A. semialata*, we observed the relationship between BS traits and mean annual temperature (MAT). Results indicate that there is no correlation between MAT and BS area (Figure 3.8 (A)). However, there is a negative correlation between MAT and the number of BS cells in each minor vascular bundle (Figure 3.8 (B)). This suggests that *A. semialata* populations originating from colder areas will have more BS cells per minor vein.

We further tested if MAT and minor vein area significantly predicted the number of BS cells using an interaction model of multiple linear regression. This test showed that the influence of MAT on the number of BS cells is explained by temperature-related variation in MV area, indicating that the increase in the number of BS cells is associated with a larger minor vein area in genotypes from colder areas.

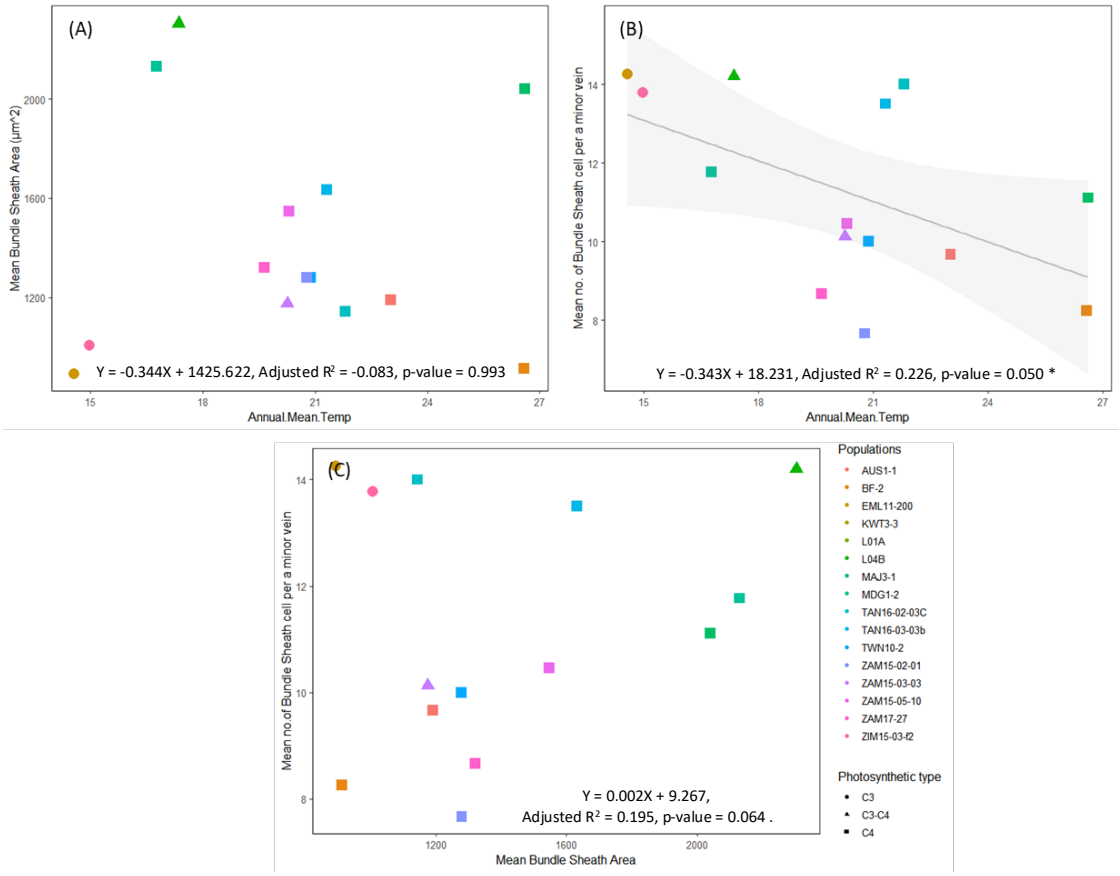

Figure 3.8 Relationship of mean annual temperature (MAT) and bundle sheath (BS) anatomical traits; (A) BS area and (B) BS cell numbers. Additionally, the relationship between BS area and its cell numbers is shown in (C).

5. Discussion

This study focused on intraspecific adaptations of leaf structures in C_4 grasses by analysing 15 genotypes of *Alloteropsis semialata* with varying geographic origins. Our findings suggest that *A. semialata* from colder climates have evolved larger minor veins, consistent with previous studies on interspecific adaptations among 26 grass species (see Chapter 2 for more details). We further investigated whether phloem or xylem tissues in minor veins are related to the size of the minor vein and adaptation to low temperatures. Results indicate that both xylem and phloem areas, as well as the number of cells, are positively correlated with minor vein area. This suggests that larger minor veins have more conduits in both xylem and phloem. However, our results indicate that only phloem is involved in adaptation to low temperatures, while xylem is not.

Phloem is a part of the vascular tissue responsible for sugar translocation from source tissues, such as leaves, where photosynthesis occurs, to sink tissues, where photosynthetic products are stored or utilised in growth or respiration. Efficient and controlled sugar distribution is essential for plant growth (Lemoine et al., 2013). However, low temperatures can slow down source-to-sink transport due to the impairment of proteins involved in assimilate loading from leaves to phloem, such as SUTs (Sucrose Transporters) (Takahashi et al., 2017) and SWEETs (Sugars Will Eventually be Exported Transporters) (Gautam et al., 2022). Additionally, the symplastic loading pathway seems to be strongly inhibited at low temperatures (Van Bel, 1993). As a result, plants in colder climates tend to use apoplastic loading, which requires more energy investment to transport sugar (Sowiński et al., 2001). The mode of phloem loading and minor vein configuration are correlated with climatic zones, with apoplastic phloem loading being dominant in temperate and arid regions, while symplastic phloem loading is dominant in tropical rainforests (Gamalei, 1989; Van Bel et al., 1992; Van Bel, 1993). Hence, the maintenance of phloem loading is vital for plants to survive at low temperatures. In our study we found that *A. semialata* from cold areas have developed a large phloem area.

To further explain how larger phloem can help plants compensate and maintain sugar loading at low temperatures, we have developed the mathematical model of phloem flow rate. According to the model, we found that if a geometric parameter of phloem cells (X) is constant, the total number of phloem cells (N) must be increased approximately five-fold to compensate for the expected effects of temperature within each phloem cell. However, among 15 genotypes of *A. semialata*, there is two-fold increase in N. Although an individual phloem cell size and radius are not significantly different among the genotypes, the range of phloem radius is between 1.9 and 2.6 μm, which can increase phloem flow rate by up to 3.5 times. In combination, the increases in both the total number of phloem cells in minor vein and individual phloem cell radius are sufficient to compensate phloem sap flow rate at low temperatures.

Our mathematical model of phloem sap flow rate involves several simplifying assumptions, which we recognise as approximations. Despite these simplifications, our model is broadly consistent with observed patterns. To further refine our model, we propose five areas of consideration:

1) Due to the limitation in the resolution of cross-sectional pictures, we could not distinguish the cell types in phloem tissue. The total area and number of cells of phloem consisted of various cell types. There are three common cell types found in minor vein phloem: sieve elements (SEs), companion cells, phloem parenchyma cells. Only SEs have a direct function in transport, whereas the others help SEs to maintain their function (Schulz et al., 2009). However, we assume that the proportions of the various cell types are fixed across genotypes. Thus, the assumption in our model is that proportional increases occur across the cell types at lower temperatures.
2) We assumed that *A. semialata* uses only apoplastic phloem loading pathway at low temperatures. This is because most grass species and Solanaceous species, including herbaceous plants, are apoplastic loaders (Slewinski et al., 2013). Moreover, chilling-tolerant maize uses an apoplastic pathway more than a symplastic pathway (Sowiński et al., 2001). However, plants can use either apoplastic or symplastic pathways or both pathways at the same time (Van Bel,

1993; Braun et al., 2009, 2014; Slewinski et al., 2013). Thus, it is possible that *A. semialata* may use a mixed phloem loading pathway.

3) We focused only on the transverse size of phloem cells and assume that length is unchanged by temperature. However, the length of SEs is an important factor that should be taken into account because it can affect hydraulic pressure in SEs and phloem flow rate (Jensen et al., 2013).
4) Sieve pores (SPo) are the principal components of phloem and are critical for phloem functions. They can be found on sieve plates connecting SEs, and also between SEs and companion cells (CCs) (Kalmbach et al., 2019), indicating that they are involved in both transportation through SEs and sugar loading/unloading via CCs. There is evidence from conifers showing that a key adaptation to live at high altitudes is having narrower and greater number of SPo on sieve plates than angiosperms (Schröter et al., 2021). Moreover, plants living in cold and dry climates tend to have lower plasmodesmatal frequencies in the phloem of minor veins, which can indicate that they are apoplastic loaders (Gamalei, 1989; Van Bel et al., 1992; van Bel et al., 1992; Davidson et al., 2011). However, in our developed model, we did not consider the effects of the structural changes and the number of SPo at low temperatures.
5) We assumed that sucrose is the only type of sugar that is transported in *A. semialata*. In fact, plants can transport photosynthetic products in several forms, such as polyols (mainly sorbitol and mannitol) and oligosaccharides of the raffinose family (Rennie et al., 2009). Nonetheless, no research is mentioned about forms of sugar that *A. semialata* used for transportation.

Xylem is a principal component of vascular tissue along with phloem. Its main roles are transporting water and solutes from root to leaves via the stem, and also providing mechanical support and storage of carbohydrates (Sack et al., 2013; Růžička et al., 2015). Water is vital to plants as it is the main component of cytosol, where various aspects of cellular metabolisms occur. Moreover, plant hydraulic conductance

in xylem is associated with photosynthetic efficiency (Brodribb et al., 2000). Thus, the maintenance of water transportation is crucial for plants to maintain their functions and survive when they grow under suboptimal conditions like low temperatures. At sub-zero temperatures, ice forms in plant cells, including xylem vessels, trapping air bubbles inside the ice crystals. During thawing, the undissolved bubbles merge to form larger ones, which can block the xylem, disrupting water transport through the vessels (Venturas et al., 2017). Because embolism is the main cause of the interruption of water transport at low temperatures, several trees and grasses living in temperate and cold regions such as conifers (Davis et al., 1999; Pittermann et al., 2006), barley (*Hordeum vulgare*) (Tamang et al., 2022), and Scutch grass (*Cynodon dactylon*), which is a C_4 grass living at high-altitude regions (Ahmad et al., 2016), have developed small metaxylem vessels to increase hydraulic pressure and maintain water transport to survive low temperatures. Additionally, having a narrow metaxylem needs less time and a smaller amount of water to refill compared to wider ones (Vesala et al., 2003). Although various findings are supporting the adaptations in the xylem and its conduits to low temperatures, our results do not show these expected temperature effects on the xylem area and metaxylem vessel size in *A. semialata*. This is in contrast with our previous study (Chapter 2), where we found that temperate grasses have developed smaller metaxylem across different lineages, which is consistent with other research. However, based on climate data of all genotypes of *A. semialata* in this study, the range of minimum temperature of coldest month (Min.TCM) is 1 - 19 ºC (Supplementary: Table S3.1). This means that only populations at the range limits for this species typically experience freezing events in nature, explaining why they do not need this adaptation. This implies that the development of small metaxylem vessels may be specifically associated with freezing rather than chilling adaptation.

Bundle sheath is where photosynthesis occurs in leaves with the C_4 photosynthetic type. At low temperatures, photosynthesis slows down due to reductions in gene expression and enzyme activities (Wang et al., 2008; Long et al., 2013). Thus, to maintain their photosynthesis during periods of low temperature, C_4 plants are hypothesised to need larger bundle sheath area so they can have more room for photosynthetic enzymes like RubisCo (Kubien et al., 2004). However, our result does not show the relationship of temperature effect and bundle sheath area, which is not

consistent with interspecific adaptation from our previous study (Chapter 2). Nevertheless, we found that the number of bundle sheath cells increases in *A. semialata* genotypes from cold regions, leading to the possibility that the increase in the numbers of bundle sheath cells may be responsible for the bigger minor vein area. However, we have not found that there is a statistical relationship between the number of cells and temperature and minor vein area. Thus, either the increase in the number of bundle sheath cells is responsible for the bigger minor vein area, or vice versa.

6. Conclusions

In this study, we examined the intraspecific adaptations of C_4 leaf anatomy to low temperatures by using *A. semialata*, a grass species with a diverse range of geographical origins, as a model plant. Our primary objective was to investigate whether the intraspecific leaf adaptations in *A. semialata* are consistent with the interspecific adaptations we observed among C_4 grass lineages in our previous study (Chapter 2). Our results show that *A. semialata* genotypes inhabiting colder areas have developed larger minor veins, which correspond to an increase in phloem tissue area and larger phloem cell numbers. These adaptations imply structural changes to compensate for the impairment of sugar loading and nutrient uptake at low temperatures. The maintenance of these processes is essential for a plant to maintain or continue its growth and metabolism processes like photosynthesis under suboptimal conditions. We also observed that xylem and bundle sheath areas, as well as metaxylem size, do not differ among various *A. semialata* genotypes. This contrasts with our previous findings (Chapter 2) and suggests that intraspecific leaf adaptations to low temperatures may differ between grass species or depend on whether freezing is experienced in nature. Nevertheless, common adaptations that help grasses tolerate low temperatures exist among different lineages.

7. Acknowledgements

I would like to thank Dr Matheus E. Bianconi and Vanja Milenkovic, for preparing leaf samples in resin blocks and producing slides of leaf cross-sections. This work was funded by a studentship awarded by the Royal Thai Government

8. Supplementary data

Table S3.1 Geographical and climate details of 15 genotypes of *A. semialata* in this study

Genotypes	Photo. type	Ploidy	Country	Latitude	Longitude	MAP (mm)	MAT (°C)	Max.TWM (°C)	Min.TCM (°C)
AUS1-1	C_4	2x	Australia	-19.62	146.96	1042	23.02	31.37	11.89
BF-2	C_4	2x	Burkina Faso	10.85	-4.83	1058	26.58	34.22	18.54
EML11-200	C_3	2x	South Africa	-26.29	30.00	761	14.56	24.18	0.52
L01A	C_3- C_4	2x	Tanzania	-5.63	32.69	934	23.50	32.44	14.79
L04B	C_3- C_4	2x	Tanzania	-8.51	35.17	1469	17.35	24.94	9.29
MAJ3-1	C_4	2x	Madagascar	-15.67	46.37	1665	26.61	33.79	17.09
MDG1-2	C_4	6x	South Africa	-25.76	29.47	670	16.77	26.41	2.47
TAN16-02-03C	C_4	2x	Tanzania	-9.04,	32.48	995	21.81	29.36	12.62
TAN16-03-03b	C_4	2x	Tanzania	-9.04	32.48	994	21.32	30.86	12.16
TWN10-2	C_4	2x	Taiwan	24.47	120.72	1994	20.88	30.10	10.75
ZAM15-02-01	C_4	6x	Zambia	-10.18,	30.95	1322	20.78	31.14	10.23
ZAM15-03-03	C_3- C_4	2x	Zambia	-10.23,	29.83	1393	20.26	30.15	9.51
ZAM15-05-10	C_4	2x	Zambia	-11.45,	29.02	1229	20.31	30.63	9.01
ZAM17-27	C_4	6x	Zambia	-17.03	26.70	752	19.65	31.66	5.02
ZIM15-03-f2	C_3	2x	Zimbabwe	-18.78	32.74	1168	14.97	23.05	4.66

Note: Abbv.: Photo. Type, photosynthetic type; Max.TWM, Maximum Temperature of Warmest Month; and Min.TCM, Maximum Temperature of Coldest Month

Table S3.2 19 bioclimatic variables downloaded and extracted from the public climatic data on the website WorldClim (Fick et al., 2017)

Codes	Variable names	Formula
BIO01	Annual Mean Temperature (MAT)	-
BIO02	Mean Diurnal Range	Mean of monthly (max temp - min temp)
BIO03	Isothermality	(BIO2/BIO7) (×100)
BIO04	Temperature Seasonality	standard deviation ×100
BIO05	Max Temperature of Warmest Month	-
BIO06	Min Temperature of Coldest Month	-
BIO07	Temperature Annual Range	BIO5-BIO6
BIO08	Mean Temperature of Wettest Quarter	-
BIO09	Mean Temperature of Driest Quarter	-
BIO10	Mean Temperature of Warmest Quarter	-
BIO11	Mean Temperature of Coldest Quarter	-
BIO12	Annual Precipitation (MAP)	-
BIO13	Precipitation of Wettest Month	-
BIO14	Precipitation of Driest Month	-
BIO15	Precipitation Seasonality	Coefficient of Variation
BIO16	Precipitation of Wettest Quarter	-
BIO17	Precipitation of Driest Quarter	-
BIO18	Precipitation of Warmest Quarter	-
BIO19	Precipitation of Coldest Quarter	-

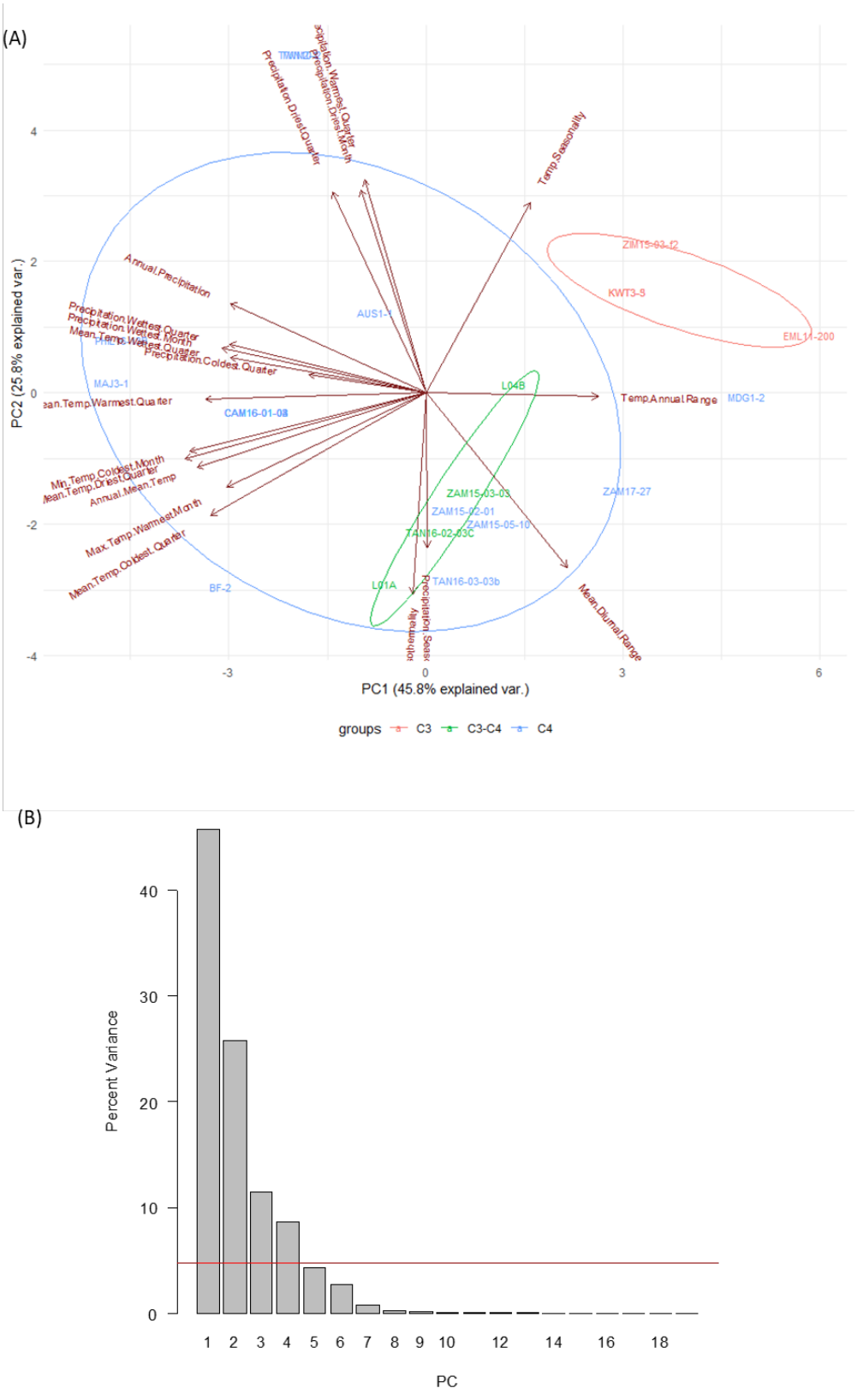

Figure S3.1 PCA analysis of 19 climate factors (Table S5. 1) among different 15 genotypes of *A. semialata*; (A) PCA plot and (B) scree plot.

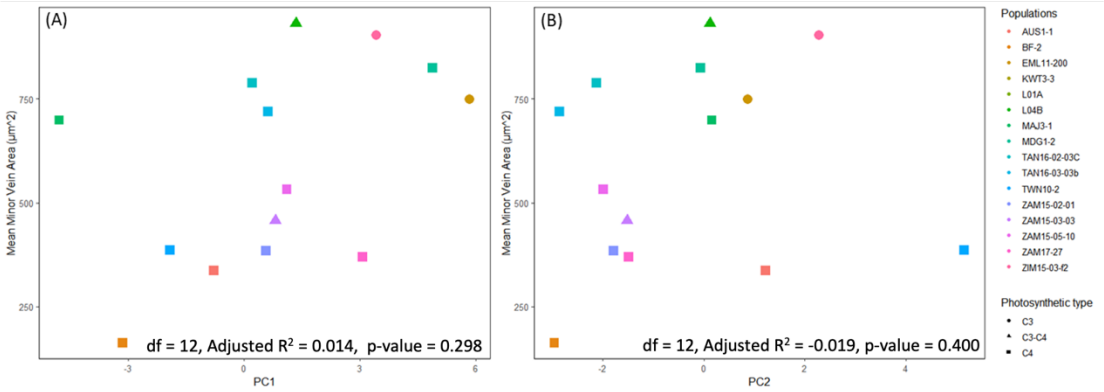

Figure S3.2 Relationship between minor vein area (µm^2) and principal component (PC) 1 and 2 from the PCA analysis of 19 climate factors among different 15 genotypes of *A. semialata*

Table S3.3 the calculation for phloem anatomical traits at 25 and 15 °C

Phloem traits	**Equation for calculation**	**MAT**	
		25 °C	**15 °C**
Ph_{area} (µm^2)	$y = -17.333x + 657.930$	224.605	397.935
N	$y = -1.213x + 40.976$	10.600	22.780
Ph_{cell_area} (µm^2)	$y = -0.321x + 23.116$	15.091	18.301
Ph_{cell_radius} (µm)	$y = (-1.195 \times 10^{-3})x + 2.427$	2.397	2.041

Note: Equations for calculation of each phloem anatomical trait obtained from PGLS analysis testing the relationship of each phloem trait and mean annual temperature (MAT) (Figure 3.4 and Figure 3.5). In each equation, y is each particular phloem trait, whereas x is MAT. Abbv.: Ph_{area} and N the total phloem tissue area and number of phloem cell per minor vein. Ph_{cell_area} and Ph_{cell_radius} represent individual phloem cell area and radius, respectively.

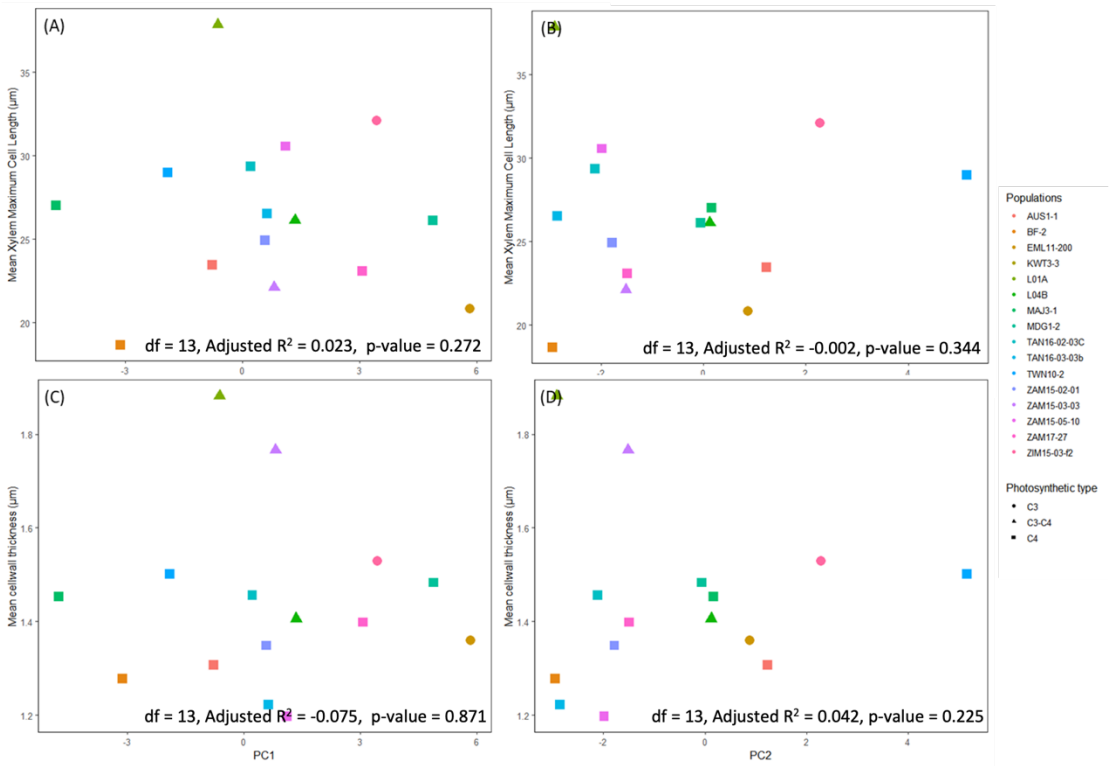

Figure S3.3 Relationship between metaxylem traits; (A-B) mean maximum cell length (MCL) and (C-D) mean cell wall thickness, and principal component (PC) 1 and 2 from the PCA analysis of 19 climate factors among different 15 genotypes of *A. semialata*

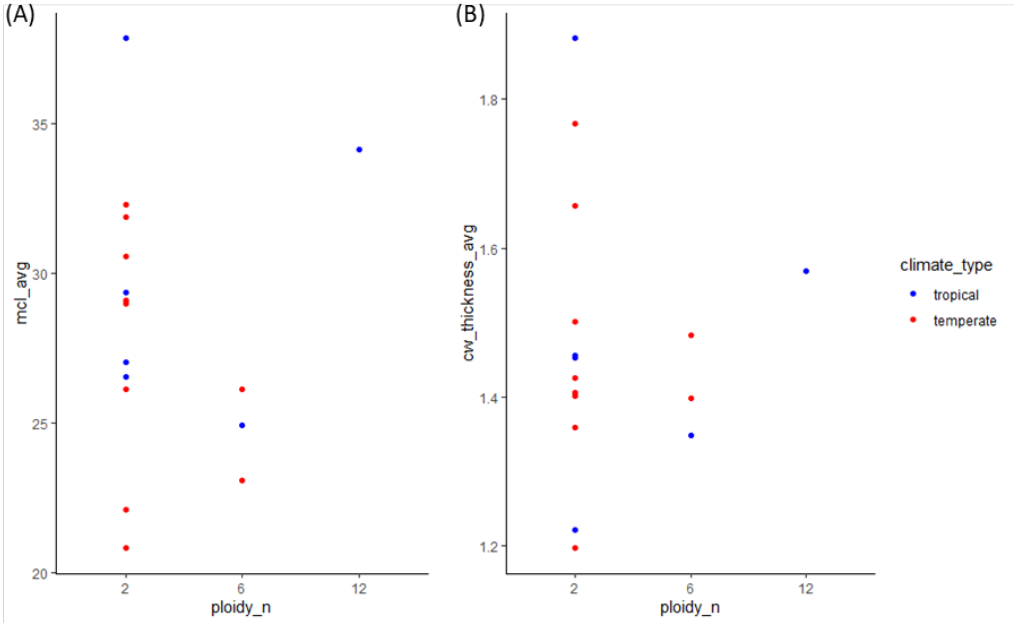

Figure S3.4 Relationship between metaxylem traits and ploidy level; (A) mean maximum cell length (MCL) and (B) mean cell wall thickness.

Chapter 4

Transcriptomic responses to short-term chilling in C_3 temperate and C_4 tropical grasses

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Personal contribution:

I co-designed the study with Prof. Colin Osborne, Dr. Pascal-Antoine Christin, and Dr. Luke Dunning. The chilling experiment was performed by Dr. Teera Wacharamonkol, a former PhD student, with Prof. Colin Osborne. Finally, I performed all analyses, interpreted the results, and wrote the paper with the help of all authors.

1. Abstract

Low temperature is a critical abiotic factor that profoundly affects plants, influencing their growth, persistence, and geographic distribution on a global scale. Within the grass family (Poaceae), the subfamily Pooideae has successfully migrated to temperate and arctic regions, demonstrating their ability to cope with freezing conditions. Although Pooideae all use the C_3 pathway for photosynthesis, some members of this family are adapted to grow in tropical and subtropical climates. Conversely, numerous C_4 grass species from the PACMAD clade, that originated from hot climates, have migrated to colder areas. Given the presence of Pooideae and PACMAD grasses in low-temperature regions it is possible they share a common mechanism for responding to and coping with the cold. To investigate this hypothesis, we conducted a comparative analysis of transcriptomic changes in the leaves of three grass species subjected to short-term chilling treatment: a C_3 temperate Pooideae (*Beckmannia syzigachne*) and two distantly related C_4 tropical grasses from the subfamilies Panicoideae (*Cenchrus brownii*) and Chloridoideae (*Sporobolus consimilis*). Our results demonstrate that each species exhibits distinct transcriptional responses to low temperatures. However, we did identify five gene groups with common expression patterns across all three species, which include genes with functions relating to the reduction of reactive oxygen species (ROS) scavenging, induction of photoinhibition in photosystem II resulting in decreased photosynthetic efficiency, inhibition of cellular and chloroplast gene expressions, as well as impacts on plant growth and development. This study suggests that C_3 temperate Pooideae grasses may share some mechanisms in response to the cold and may likely share some mechanisms with distantly related tropical C_4 species. Nevertheless, they have also developed distinct mechanisms to adapt and survive in low-temperature environments, indicating their unique evolutionary trajectory.

2. Introduction

Low temperature is a significant abiotic factor affecting the biochemistry and physiology of plants (Janská et al., 2010; Theocharis et al., 2012; Rihan et al., 2017; Szymańska et al., 2017; Ritonga et al., 2020; Bhattacharya, 2022). It acts as a primary limiting factor influencing plant performance, growth, and development (Wingler et al., 2016), as well as determining their geographical distribution on a global scale (Woodward, 1988, 1990). Exposure to low temperatures leads to a deceleration of biomolecular mechanisms, such as chemical reactions and transcription, within plants, resulting in reduced efficiency across various physiological processes, including photosynthesis (Hendrickson et al., 2004), respiration (ap Rees et al., 1988; Labate et al., 1989b), water transport (Sucoff, 1969), and translocation (Weatherley et al., 1969; Webb, 1971; Farrar, 1988).

Surviving at low temperatures is challenging for plants as they are unable to move. Plants need to adapt themselves to deal with the effects of exposure to low temperatures by changing gene expression which then induces biochemical and physiological modifications to enhance their tolerance and prevent damage. This process is called chilling or cold acclimation (Smallwood et al., 2002; Theocharis et al., 2012). Those modifications are involved in various changes in diverse mechanisms such as modification in plasma membranes and cell walls (Campos et al., 2003; Cruz et al., 2010; Tian et al., 2022), photosynthetic acclimation to maintain photosynthetic efficiency (Kubien et al., 2003; Naidu et al., 2004; Zhang et al., 2004; Long et al., 2013), accumulation of reactive oxygen species (ROS) and activation of the scavenging system to prevent photodamage and photoinhibition (Szymańska et al., 2017), and maintaining sugar translocation in phloem (Gamalei, 1989; Van Bel et al., 1992; Davidson et al., 2011; Schröter et al., 2021). However, the mechanisms different species use to cope with low temperatures can vary depending on their physiology and evolutionary history.

The grass family (Poaceae) has successfully migrated to cold temperate regions (Hopkins et al., 2006; Linder et al., 2018). Within 12 subfamilies of Poaceae, the subfamily Pooideae stands out as a well-known group of grasses that dominate temperate and arctic floras (Schubert et al., 2019). These grasses have adapted

physiologically to endure extreme environmental conditions like seasonal changes, and frost (Schubert et al., 2019). Various species within the Pooideae have demonstrated their ability to undergo cold acclimation such as *Brachypodium distachyon* (Li et al., 2012) and *Deschampsia antarctica* (Olave-Concha et al., 2004). During this process, five main gene families play crucial roles in response to cold and cold acclimation: C-repeat-binding factors (CBF; (Badawi et al., 2007)), dehydrin (DHN; (Olave-Concha et al., 2004; Kosová et al., 2007, 2010)), chloroplast-targeted cold-regulated proteins (ctCOR; (Crosatti et al., 1999, 2013)), ice recrystallisation inhibition proteins (IRIP, (John et al., 2009)) and fructosyl transferases (FST; (del Viso et al., 2009; Tamura et al., 2014)).

Although Pooideae species dominate the northern temperate and arctic grass floras (Clayton, 1981), the ancestors of this subfamily were most likely adapted to tropical and subtropical climates (Bouchenak-Khelladi et al., 2010). Moreover, Watcharamongkol, Christin and Osborne (2018) have shown that C_4 grass lineages have migrated more frequently into cold environments than their C_3 counterparts. There are numerous examples of C_4 grasses successfully migrating to grow in temperate and high-altitude regions, including Bermuda grass (*Cynodon dactylon*) (Ahmad et al., 2016) and *Cleistogenes squarrosa* (Liu et al., 2008). These suggest that the successful migration of Pooideae to low-temperature regions may, in part, enabled by ancestral cold-tolerance mechanisms that are shared with extant C_4 PACMAD grasses. However, study of this question remains limited, necessitating further research to uncover the mechanisms underlying the remarkable success of Pooideae as a grass subfamily in migrating to cold temperate regions.

In this study, we conducted RNA sequencing to identify cold-responsive genes in three grass species for short-term chilling treatment. Specifically, the gene expression profiles of *Beckmannia syzigachne*, a C_3 temperate Pooideae species, were contrasted with those of two distantly related tropical C_4 species, namely *Cenchrus brownii* and *Sporobolus consimilis*. Our primary objective was to identify differentially expressed genes (DEGs) and gain insights into their functional implications. By analysing the obtained data within a phylogenetic framework, we aimed to determine whether there is convergence among cold-responsive genes in diverse species adapted to cold and warm environments. Additionally, we sought to investigate the presence of shared or

distinct gene expression patterns and mechanisms in response to low temperatures among tropical grasses and between temperate and tropical grasses (Figure 4.1).

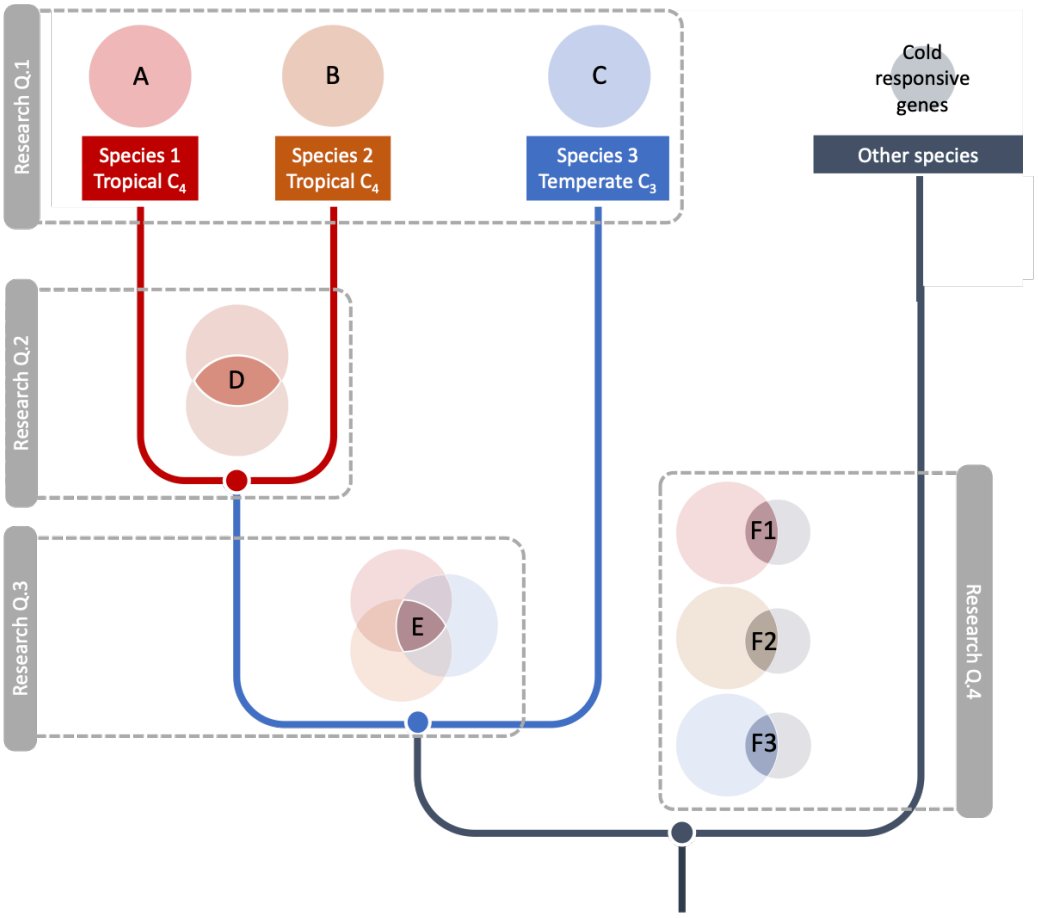

Figure 4.1 The establishment of research questions in this transcriptomic study. The letters in the diagram represent different groups of expressed genes associated with specific aspects. Group 'A' and 'B' represent genes expressed in particular tropical C_4 species, while group 'C' represents expressed genes in temperate C_3 grass. Group 'D' comprises genes that are commonly expressed in tropical C_4 grasses in response to low temperatures. Group 'E' represents genes that are expressed in all three species, encompassing both tropical and temperate species. These genes are involved in cold responses inherited from tropical ancestors. Finally, group 'F' represents the cold-responsive genes observed in other plant species and presented in the grass species present in this study.

3. Methods

Plant materials, treatments, and experimental design

Two C_4 distantly related tropical grasses, *Cenchrus brownii* (CBR) and *Sporobolus consimilis* (SCO) from clade PACMAD, together with *Beckmannia syzigachne* (BSY) from a clade BOP, a C_3 temperate grass, were studied in this study (Figure 4.3). The chilling treatment and plant material were generated by Dr Teera Wacharamonkol, a former PhD student supervised by Prof. Colin Osborne. Briefly, the plants were grown from seeds and cultivated in separate containers within a controlled environment chamber comprising a 12-hour light cycle with a photon flux density (PFD) of 600 µmol m^{-2} s^{-1}, measured at the height of the plants. The day/night temperature was maintained at 25/20 °C with a relative humidity of 60/80%. Plants were acclimated under these conditions for 12 weeks.

The temperature experiments were carried out for 14 days with the same growth conditions during the acclimation period except for the temperature (Figure 4.2). For the control treatment, plants were grown under a day/night temperature of 25/20 °C, while the plants with chilling treatment were treated at a day/night temperature of 15/5 °C. Leaf samples were collected from both chilling and control treatments on the day 14^{th} after starting the chilling treatment. The samples were flash-frozen in liquid nitrogen before being stored at -80 °C for RNA extraction.

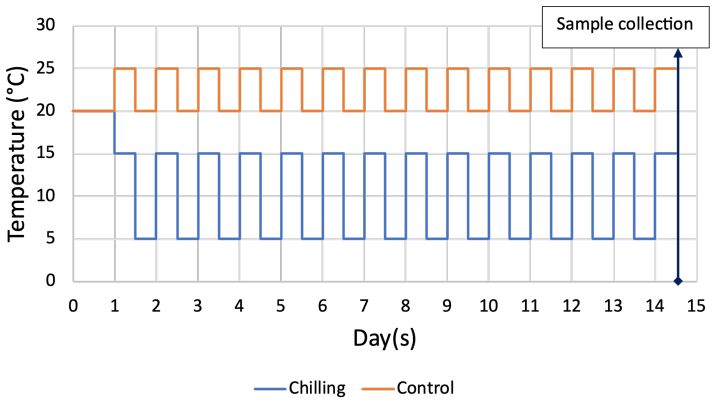

Figure 4.2 Experimental design for chilling treatment. The chilling treatment was treated with day/night temperatures of 15/5 ºC and the control treatment was treated with day/night temperatures of 25/20 ºC for 14 days. (Adapted from Watcharamongkol (2019)).

RNA extraction, library preparation, and RNA sequencing

RNA samples were extracted from approximately 10 leaf samples per biological replicate of each species and using the RNeasy® Plant Mini Kit following the manufacture's protocol (QIAGEN, Valencia, CA, USA). Extracted RNA was treated with RNase-Free DNase set (QIAGEN, Valencia, CA, USA) to remove genomic DNA. The total RNA integrity was accessed by gel electrophoresis with 0.8% Agarose in 1x Tris-Borate-EDTA (TBE) buffer mixed with Ethidium bromide (EtBr) to a final concentration of approximately 0.5 µg/mL. The quantity and purity of RNA were assessed in a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

RNA library preparation and RNA sequencing were performed by the Oxford Genomics Centre (OGC) (Oxford, UK). In brief 18 RNA samples (3 species × 2 temperature treatment × 3 biological replicates) were prepared for RNA-sequencing (RNA-seq) before being paired-end sequenced using two units of a flow cell of an Illumina NovaSeq 6000.

De novo transcriptomic assembly and alignments

The quality of raw Illumina sequencing reads was assessed using FastQC. Adapter sequences were removed using Trimmomatic v. 0.39 (Bolger et al., 2014) with default parameters and this was verified using FastQC. *De novo* transcriptome assemblies were generated from each species using Trinity v.2.11.0 (Grabherr et al., 2011). Sequence alignments were done with BOWTIE2 v.2.3.4.3 (Langmead et al., 2012) with default parameters against the transcriptome of the corresponding species. SAMtools v. 1.15 (Li et al., 2009) with default parameters was used for downstream processing of the alignments to generate raw expression counts.

RNA-Seq Transcript Analysis

(1) Gene Ortholog Finder and Gene annotation

ORF Finder v. 0.4.3 was used with default parameters to search for open reading frames (ORFs) that can be potentially encoded for protein in the assembled transcripts. The transcript ORFs were then used as input for inferring the corresponding orthologs of the grasses OrthoFinder v. 2.5.2 (Emms et al., 2019) with default parameters. In this study, we used mouse-ear cress (*Arabidopsis thaliana*) and seven closely related grass species which are stiff brome (*Brachypodium distachyon*), rice (*Oryza sativa*), *Oropetium thomaeum*, Switchgrass (*Panicum virgatum*), great millet (*Sorghum bicolor*), *Setaria italica*, and maize (*Zea mays*), and one outgroup species, Pineapple (*Ananas comosus*) (Figure 4.3). To annotate the functions of each transcript, the blastn program in BLAST+ package v.2.14.0 was used by using *A. thaliana* from the UniProt database as a reference.

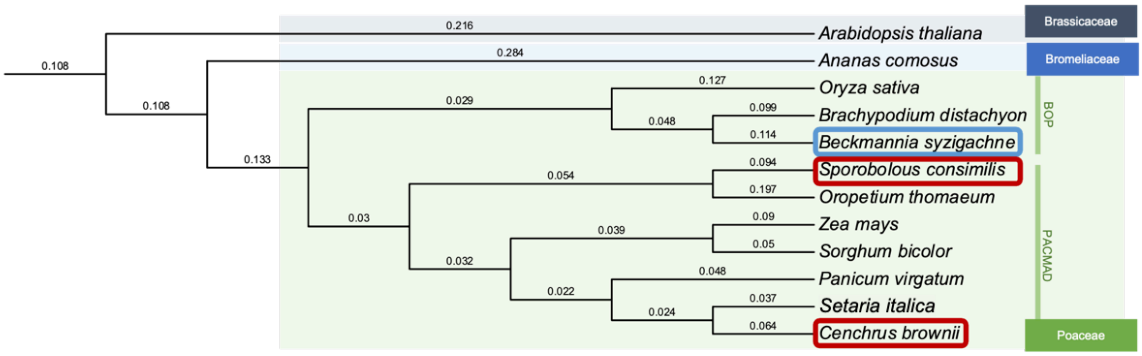

Figure 4.3 Phylogenetic tree of 12 species that were used in the ortholog analysis by OrthoFinder. Three species in blue and red rectangles are tested species. Blue is a C_3 temperate grass, red represents C_4 tropical grasses. This tree was generated online by iTOL version 6.7.6.

(2) Differentially gene expression (DEG) analysis

To compare the gene expression levels between control and chilling-treated groups of particular species, firstly, eXpress v.1.5.1 (Roberts et al., 2013) was used to quantify RNA-seq data by counting reads for each unique transcript in each particular sample. Then, empirical analysis of digital gene expression (*edgeR*) (Robinson et al., 2010), a Bioconductor package in R, was used to normalise and do the differential expression analysis of sequence read counts between control and chilling treatments in each species. For each comparison, we used a false discovery rate (FDR) of 0.05 as the cut-off value.

(3) Gene ontology (GO) and pathway enrichment analysis

To investigate whether the DEGs were associated with any particular metabolic functions a gene ontology (GO) pathway enrichment analysis was performed. The GO enrichment analysis was done by using *clusterProfiler* 4.0 package in R (Wu et al., 2021) by using *enrichGO()* function. All genes from each transcriptome were used for this analysis. The p-value adjustment method was set to "BH" (Benjamini & Hochberg,

same as “fdr”) (Haynes, 2013) and the p-value and *q*-value cut-off were set at 0.05 and 0.05, respectively. The Universal Protein Resource (UniProt) IDs were changed to The Arabidopsis Information Resource (TAIR) ID before using *enrichGO()*.

(4) Comparison of multiple genes between different species

To compare multiple gene groups between different species, the *comparecluster( )* function in *clusterProfiler* 4.0 package was used. *Arabidopsis thaliana* was used as an organism database and the function was set as “enrichGO”. Notably, the all-tested genes from DEG analysis including genes that do not pass the criteria of this analysis (p-value > 0.05) were used in this pathway analysis. The other parameters were set as default.

(5) Searching for cold-responsive and conserved genes reported from other species

To assess the presence of cold-responsive genes and conserved genes, especially those reported in other plant species, specifically grasses, we searched for specific genes. This search encompassed three genes encoding photosynthetic enzymes (PEPC, PPDK, and RubisCo), as well as genes from five key gene families pivotal in responding to cold stress and cold acclimation (CBF, COR, DHN, IRIP, and FT) (Schubert et al., 2019) from *Arabidopsis thaliana* and six species of grasses, which are maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), perennial ryegrass (*Lolium perenne*), and timothy grass (*Phleum pratense*). The BlastN program within the BLAST+ package v.2.14.0 was used to query these genes against the transcriptomic sequences of each species under investigation. The E-value was set at 1e-10 and a maximum of five target sequences were considered. The list of the testing genes and their accession number is shown in Table S4.4.

To assess the gene expression levels of these transcripts, we analysed log fold change (logFC) and log counts per million (logCPM) by cross-referencing the results obtained from the eXpress program.

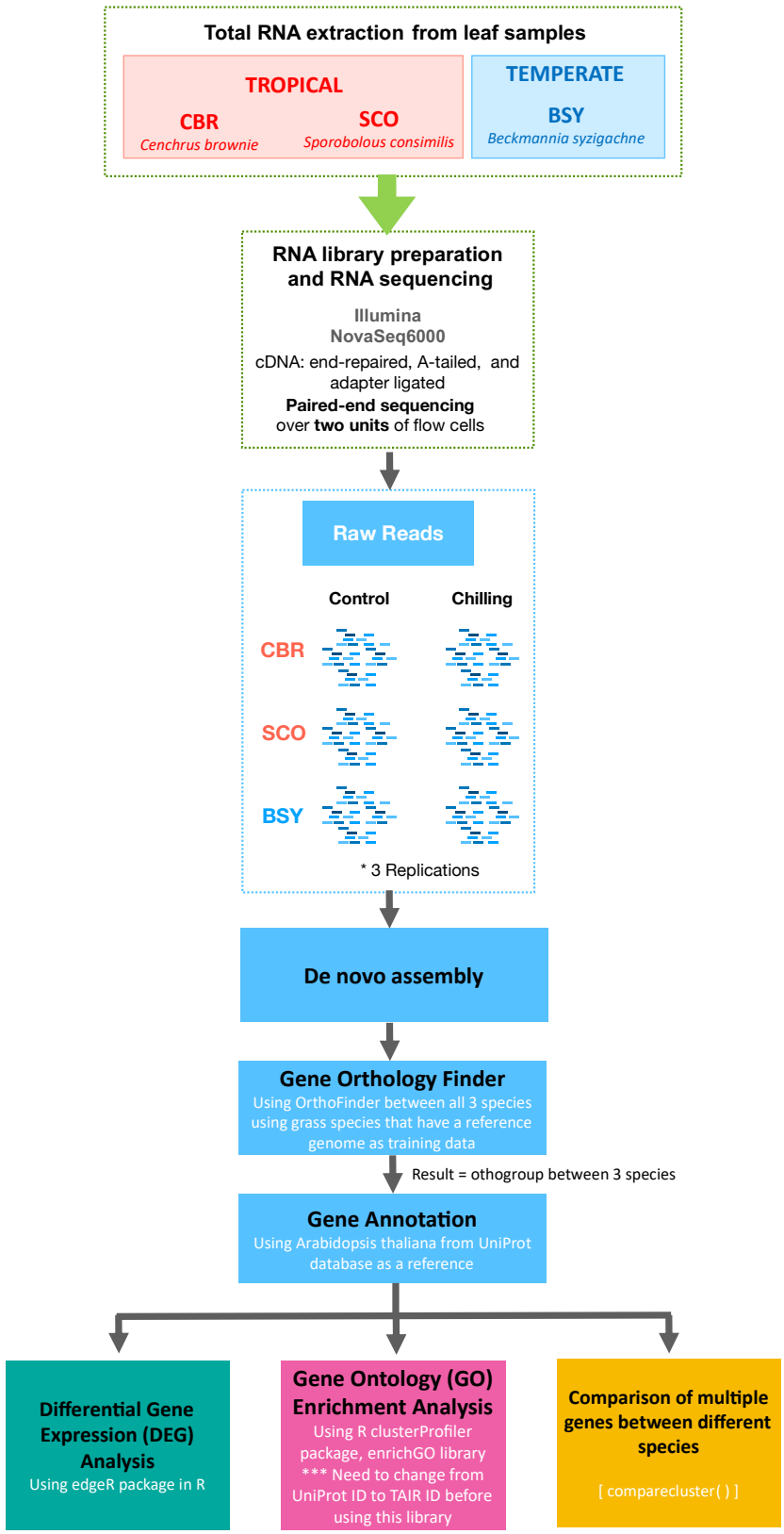

Figure 4.3 The overview of this study, transcriptomic analysis of C_4 grasses to chilling temperatures.

4. Results

Sequencing and read assembly

After the RNA sequencing, we obtained the total number of raw reads, combining both forward and reverse directions of sequencing, from all samples with means 4,982,546 ± 2,022,666 (Supplement: Figure S4.2 (A) and Table S4.1). Most samples have total raw reads of more than 10,000,000 except for the three samples from SCO (SCO_C1, SCO_T1, and SCO_T4). In addition, after the quality of raw reads was assessed by FastQC, all samples from SCO except SCO_C4 show a percentage of low-quality reads > 25% (Supplement: Figure S4.2 (B)). In combination with RIN values, this implied that most of the samples from SCO have low-quality RNA raw material resulting in a smaller number of reads that can pass the quality assessment by FastQC (Supplement: Table S4.2).

We further assembled the obtained cleaned reads to build a transcriptome for each particular species by Trinity. After this step, CBR shows the highest assembled transcripts, totalling 551,972, nearly twice the count observed in BSY and SCO (Supplement: Table S4.3). Approximately half of the transcripts in each particular species could not be annotated by using *A. thaliana* as a reference, leaving their gene functions unknown (Supplement: Table S4.3).

Differentially Expressed Genes (DEGs) analysis

In DEG analysis, the gene expression profiles of each particular biological replicate were accessed and compared by using a multidimensional scaling plot to check the consistency of the expression profiles among each particular species. The result shows that the gene expression profiles of control and chilling-treated samples in both CBR and BSY are clearly separated (Supplement: Figure S4.1 (D) and (G)), whereas there are two replicates from both control and treatment groups that cluster together in SCO (Supplement: Figure S4.1 (A)). This implies that both control and treatment groups of SCO may not show much difference in gene expression pattern. This implication is confirmed by Smear plots showing the significant differences in DEGs of each species which show that SCO has a smaller number of DEGs than CLO and BSY (Supplement: Figure S4.1 (C), (F), and (I)). Closely looking into the number of DEGs (Table 4.1), SCO has 104 DEGs, 55 up-regulated and 49 down-regulated genes, which is lower than the other two species, CLO and BSY with DEGs of 499 and 187, respectively. In addition, the number of genes with no significant differential expressed in SCO (3,953 genes) is higher than the other species about 9-14%.

Table 4.1 DEGs results showing the patterns of gene expression profiles of those three species in this study.

Species	**Total number of tested genes**	**Number of expressed genes (p-value < 0.05)**	
		Upregulated	**Downregulated**
SCO	4,057	55	49
CBR	4,123	282	217
BSY	3,669	114	73

General mechanisms in tropical C_4 grasses responding to short-term chilling temperatures

In CBR, a C_4 tropical grass belonging to the subfamily Panicoideae, we observed the highest number of differentially expressed genes (DEGs) with a p-value < 0.05, totalling 518 genes. Among these genes, 282 were up-regulated, while 217 were down-regulated (Table 4.1). Notably, 54 genes were shared with SCO, another C_4 tropical grass from the subfamily Chloridoideae. This shared gene set consisted of 21 up-regulated genes, 19 down-regulated genes, and 14 genes expressed in opposite directions between both species (Figure 4.5 (A)).

SCO exhibited a total of 104 statistically significant DEGs with a p-value < 0.05, among which 55 genes were up-regulated and 49 were down-regulated (Table 4.1). Among these genes, 70 were uniquely expressed in SCO, and 23 genes showed common expression patterns with BSY, a C_3 temperate Pooideae grass (Figure 4.5 (A)).

In the case of BSY, we identified a total of 187 DEGs with a p-value < 0.05, comprising 114 up-regulated genes and 73 down-regulated genes (Table 4.1). Among these genes, 97 were exclusively expressed in BSY, while 26 and 6 genes were shared with CBR and SCO, respectively (Figure 4.5 (A)). Furthermore, 17 genes exhibited common expression patterns across all three species, which will be discussed in a later section.

Common cold-responsive mechanisms in the C_4 tropical grasses

In our study, we used CBR and SCO, which are two distantly related tropical C_4 species. Our results show that some gene expression patterns between both tropical grasses are different, but that some are shared. There are 71 genes shared common expression between the tropical species. Among those genes, 54 genes are expressed only in tropical grasses, while 17 genes are also expressed in BSY, a temperate C_3 species (Figure 4.5 (B)). Three main gene categories shared common expression in both tropical species, which are Uridine diphosphate (UDP) -glucosyltransferase activity, active ion transmembrane transporter activity, and glucosyltransferase activity (Figure 4.6).

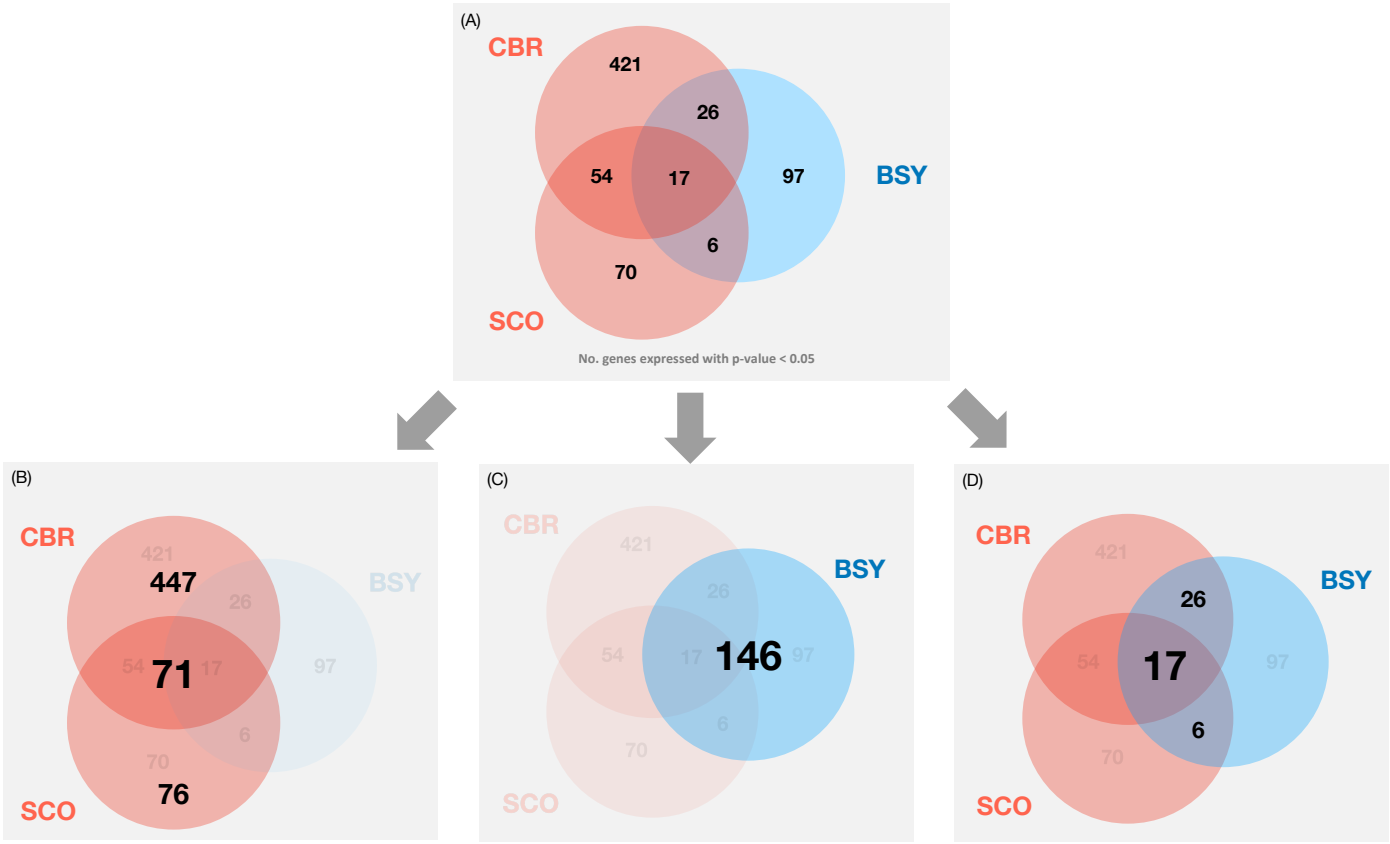

Figure 4.4 Differential gene expression analysis showing the number of genes expressed with p-value <0.05. Red indicates tropical and blue temperate species. (A) shows overall results for both genes that are uniquely expressed in particular species and commonly expressed between species. (B) show genes expressed in each tropical species. (C) shows the number of genes expressed in temperate species. (D) shows expressed genes common to tropical and temperate species.

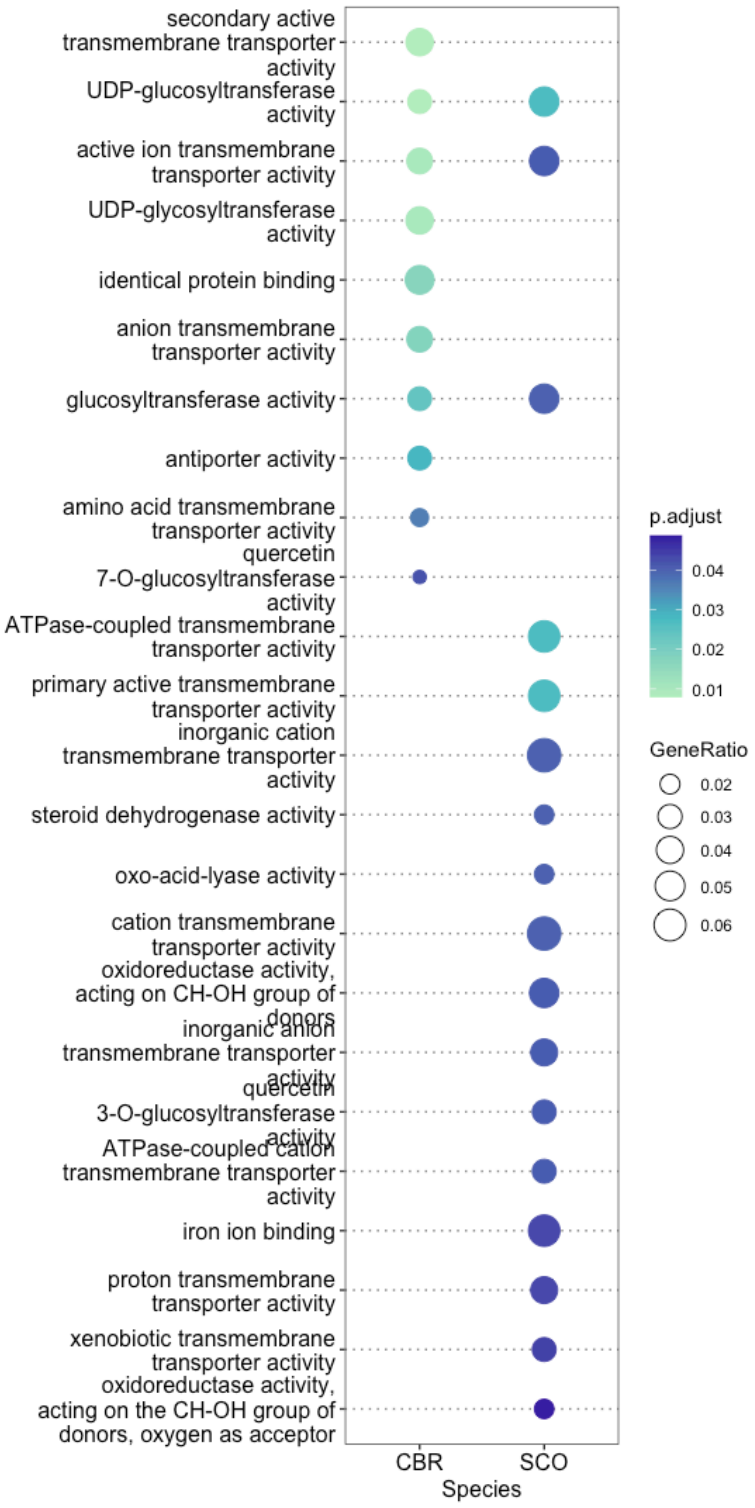

Figure 4.5 GO enrichment analysis showing gene categories comparing between two distantly related tropical C_4 species; *Cenchrus brownii* (CBR) and *Sporobolus consimilis* (SCO).

General mechanisms in temperate C_3 grasses responding to short-term chilling temperatures

Our DEG results show that 146 genes are significantly differential expressed with p-value < 0.05 in BSY, a temperate C_3 grass (Figure 4.5 (C)). Of these, 97 genes are only expressed in BSY but not in the tropical C_4 grasses, whereas 49 genes share common expressions among the temperate and tropical species (Figure 4.5 (D)), which will be covered in the next section.

From GO enrichment analysis, the differentially expressed genes in BSY (Figure 4.6) can be categorised into five main groups; (1) nucleoside nucleotide diphosphate phosphate, (2) cellular response reactive oxygen (ROS), (3) establishment of membrane organisation and localisation, (4) pigment chlorophyll biosynthesis processes, and (5) ion transmembrane transport.

Among the expressed genes in BSY, the gene group responding to cold shows the highest gene count (Figure 4.7). These genes serve multiple roles, merging with various gene sets, and showcasing their diverse functions in tackling low-temperature stress. For instance, among the 45 cold-responsive genes, five contribute to managing reactive oxygen species (ROS) responses, while another three are linked to regulating stomatal movement. Additionally, there is also overlap with genes from each of the following gene sets; generation of precursor metabolites and energy, nucleobase-containing small molecule metabolite process, ROS metabolic process and responding, response to ROS, protein targeting chloroplast, and plastid organisation (Figure 4.7). This indicates that there are various genes involved in responding to low temperatures via various mechanisms, mainly ROS metabolite process and response.

The second top highly expressed gene group are those involved in the generation of precursor metabolites and energy (Figure 4.7). Moreover, this gene set also overlaps with the other sets involved in various metabolic processes, which are Pyruvate, ROS, and pigments, and physiological responses such as plastid organisation and stomatal movement (Figure 4.7). This suggests that during growth at low temperatures, BSY may need energy to compensate for various metabolic and physiological processes that may deteriorate during low temperatures.

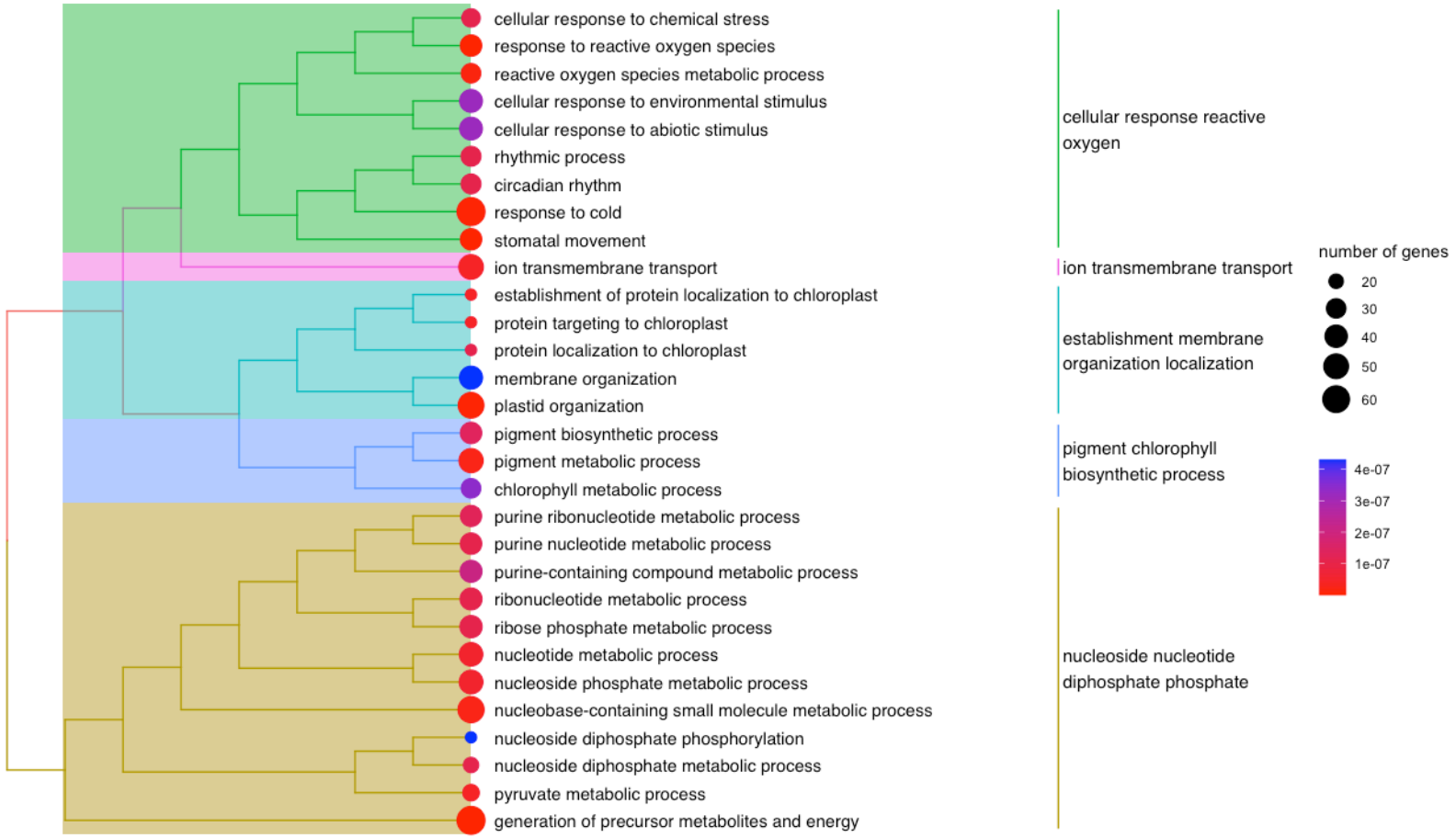

Figure 4.6 Gene category tree showing significantly enriched categories from GO enrichment analysis of *Beckmannia syzigachne* (BSY) after chilling for 14 days. The colour blue to rad indicates p-value of the analysis.

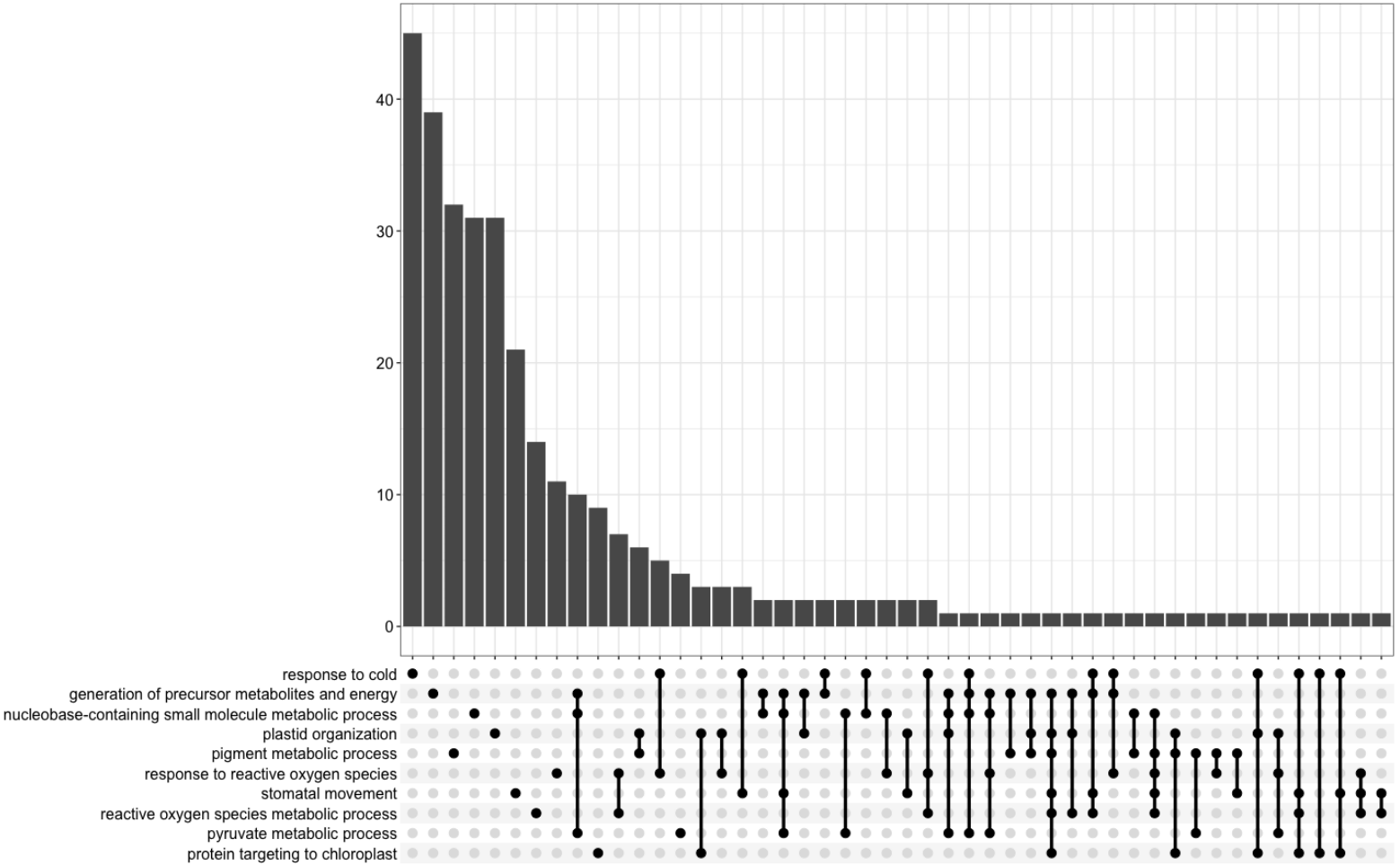

Figure 4.7 Upset plot showing the complex association between genes and gene sets emphasising the overlap among different gene sets from GO enrichment analysis of *Beckmannia syzigachne* (BSY) after chilling for 14 days. The number on the y-axis is a gene count.

C_3 temperate grass shares some common expressions with distant C_4 tropical relatives in responding to low temperatures

As mentioned above, the C_3 temperate species partially share and use the same mechanisms that are already present in distant tropical relatives, indicating a shared ancestry for these mechanisms. Our DEG analysis shows that there are 17 genes in BSY, a C_3 grass, shared between it and the other two C_4 grasses, CBR and SCO (Figure 4.5 (D)). Among these genes, there are 12 genes showing the same directions of gene expression in all three species. Eight genes are downregulated in both C_4 and C_3 species. However, both C_4 species show higher down-regulation in most of those genes, except in the SVR3 and IAA26 genes, and SCO shows less down-regulation than CBR and BSY. Four genes show up-regulated expression in all three species with similar levels of expression. Two genes are down-regulated in the C_3 species, whereas they are up-regulated in both C_4 species. Conversely, two further genes are upregulated in the C_3 species but downregulated in both C_4 species. Additionally, ANTR1 is the only gene that shows up-regulation in SCO, but down-regulated in other species (Figure 4.8).

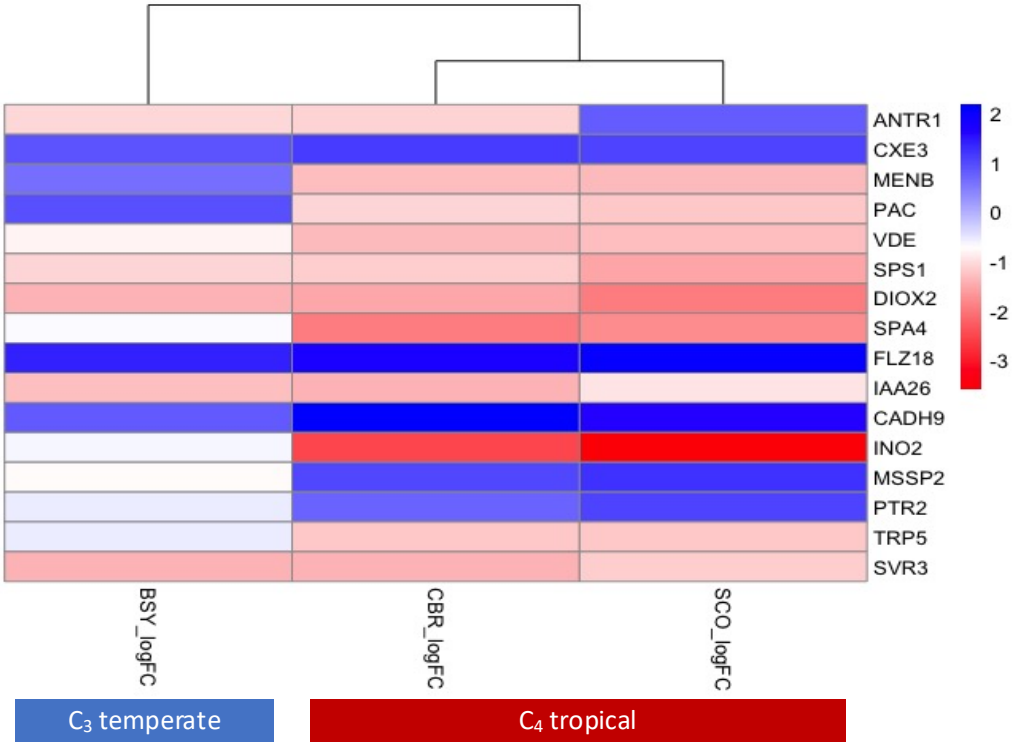

Figure 4.8 Heatmap of commonly differentially expressed genes in response to 14 days cold treatment in both C_4 tropical and C_3 temperate grasses. The names of genes are shown in UniProt Entry names (annotation and functions of each gene are shown in (Table S4.3) Gradient colour bar represents log2 fold change of each gene referring to a level of gene expression; up-regulated (blue) and down-regulated (red). Abbreviation: *Cenchrus brownii* (CBR), *Sporobolus consimilis* (SCO), and *Beckmannia syzigachne* (BSY)

Conserved genes and genes previously reported to be involved in cold responses in the species in this study

Following our stringent differential expression (DE) analysis criteria, genes with a p-value < 0.05 and genes that exhibited no expression in at least one of the three species were excluded from the analysis. Consequently, certain conserved genes, such as phosphoenolpyruvate carboxylase (PEPC), pyruvate orthophosphate dikinase (PPDK), ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCo), as well as some cold-responsive genes previously reported in other plant species, did not appear in our list of differentially expressed genes (DEGs). To address this issue, we adjusted the criteria and individually searched for each specific conserved gene and cold-responsive gene, as reported in other plant and closely related grass species, within the transcriptome of each of our studied species.

The BlastN results revealed transcripts from each species corresponding to genes encoding photosynthetic enzymes such as PEPC, PPDK, and RubisCo showing high similarity (ranging from 75% to 98%) and extremely low E-values (E-values < 8.43E-56) (Supplement: Table S4.5). Although we found the transcripts that possibly were those photosynthetic genes, some transcripts in particular species did not exhibit notable expression levels during the DEG analysis. For example, the PEPC gene in CBR and SCO, PPDK in SCO, and RubisCo in BSY and SCO (Supplement: Table S4.5).

We hypothesised that the expression level of photosynthetic genes should be decreased in both C_3 and C_4 species. As expected, the putative PEPC gene in BSY and PPDK gene in CBR were downregulated with the log fold change of -0.446 and -0.308, respectively (Supplement: Table S4.5). Surprisingly, we found that a putative RubisCo gene in CBR was upregulated with a logFC of 0.644. Moreover, a putative PPDK gene of BSY was slightly upregulated with a logFC of 0.013. However, all those expressions are not statistically significant due to a high p-value (more than 0.05).

We further searched for the five distinct groups of cold-responsive genes reported to be conserved within the core Pooideae subfamily, encompassing CBF (C-repeat/DRE binding factor), COR (cold-regulated proteins), DHN (Dehydrin), IRIP (Ice recrystallisation inhibition proteins), and FT (Fructosyl transferases) genes

(Schubert et al., 2019). Our BlastN analysis revealed the presence of most of these genes in all three species, exhibiting substantial similarity levels ranging from 77% to 88% and notably low e-values (Supplement: Table S4.5).

However certain putative genes were identified exclusively in specific species but were absent in others (Supplement: Table S4.5). For instance, while putative CBF1 genes were solely detected in BSY and CBR, both putative CBF3 and CBF4 genes were found in BSY and SCO. Additionally, putative WCS19 was present in CBR and SCO, and remarkably, putative TaIRIP was exclusively observed in BSY. This implies a potential lack of conservation of these genes across all three species, suggesting potential conservation in only particular species.

Similarly to the putative photosynthetic enzyme genes, most of the putative cold-responsive genes either did not appear in the DEG analysis or showed expression levels that were not statistically significant (p-value > 0.05) (Supplement: Table S4.5). However, we encountered unexpected findings where the putative genes from both the DHN and FT groups, expressed with a p-value < 0.05, were all downregulated. Within the DHN gene group, the putative DHN8 genes exhibited greater downregulation in BSY (logFC = -2.404, p-value = 1.60E-09) compared to CBR (logFC = -1.059, p-value = 0.048). Intriguingly, a putative WCS120 gene in CBR, which shares the same transcript as the putative DHN8 in CBR, displayed a similar trend. In the case of the FT gene group, the putative WFT2 and PpFT1 genes in CBR referred to the same transcript, showcasing significant downregulation with a logFC of -3.471 (p-value = 7.50E-07). Conversely, a putative WFT1 in BSY exhibited a milder downregulation with a logFC of -0.571 but did not reach statistical significance.

5. Discussion

Contamination Issues and Subfamily Identification Challenges

Originally, the primary objective of this study was to analyse the transcriptomic profiles of two pairs of closely related C_4 grasses from distinct subfamilies—Panicoideae and Chloridoideae—in tropical and temperate regions (Figure S4.3). The goal was to investigate gene expression changes in response to chilling stress, examining the differences in how tropical and temperate grasses within the same subfamily, as well as those from different subfamilies, adapt to such conditions. We aimed to determine whether these grasses utilise similar or distinct molecular mechanisms for adaptation, considering the varying climate origins within the same subfamily and among different subfamilies. The selection of these specific grass species was based on two factors which are sample availability and their known capacity to maintain photosynthetic efficiency at low temperatures, as evidenced in a prior study by Dr. Teera Watcharamongkol (Watcharamongkol, 2019).

In summary, the study involved selecting two pairs of tropical and temperate grasses. The temperate species exhibited minimal changes in the maximum quantum yield of photosystem II (PSII) following exposure to chilling conditions, with day/night temperatures maintained at 10/5 °C for 14 days (Fv/Fm AC14). However, during the de novo transcriptomic assemblies, issues were identified in two species. Firstly, contamination was detected in one out of three biological replicates of a species labelled *Calamovilfa longifolia* (CLO), a C_4 temperate grass from the Chloridoideae subfamily. Additionally, another species, *Pennisetum clandestinum*, was misclassified and later identified as *Beckmannia syzigachne*, a C_3 temperate grass from the Pooideae subfamily. Consequently, CLO had to be excluded from the study, leading to necessary modifications in the research questions and subsequent analyses.

Transcriptomic Analysis of Cold Response in Previously Unknown Grass Species

This study aimed to elucidate the gene expression patterns and underlying mechanisms in response to low temperatures in both C_3 temperate and C_4 tropical grass species using RNA sequencing. Given the absence of reference genomes for these species, a de novo transcriptomic approach was employed.

Upon completion of de novo transcriptomic assemblies for all three grass species, notable findings emerged. The analysis revealed a more consistent response among samples in C_3 species compared to C_4 species (Supplement: Figure S4.1). While samples from the C_4 species SCO exhibited similar gene expression patterns in two control and chilling treatment samples, the remaining samples displayed distinct separation between control and chilling treatments. This observed discrepancy possibly could be from biological factors, such as variable stress responses in plants, or potential issues during sample collection or RNA extraction processes.

Common cold-responsive mechanisms in the C_4 tropical grasses

Despite differences observed between species, our de novo transcriptomic analysis revealed shared mechanisms enabling survival in low temperatures among C_4 tropical grasses. Specifically, CBR and SCO, two distantly related tropical C_4 species, exhibited overlapping expression patterns across three key gene categories: uridine diphosphate (UDP)-glucosyltransferase activity, active ion transmembrane transporter activity, and glucosyltransferase activity. These shared gene expression profiles suggest common adaptive strategies employed by tropical C_4 grasses to cope with low-temperature stress.

The UDP - glucosyltransferase (UGT) family is the biggest glucosyltransferase (GT) family in plants involved in transferring glycosyl residues from activated nucleotide sugars to a variety of acceptor small molecules, thus regulating properties of the acceptors such as their bioactivity, solubility and transport within the cell and throughout the organism (Ross et al., 2001). UGTs can be strongly induced by various abiotic stresses, including extreme temperatures. In *Arabidopsis thaliana*, the expression of UGT79B2 and UGT79B3 is directly controlled by CBF1 (CRT/DRE-

binding factor 1 or C-repeat/drought-responsive element binding factor 1) in response to low temperatures via modulating anthocyanin accumulation (Li et al., 2017). In addition, UGTs in the tea plant (*Camellia sinensis*) are involved in the regulation of flavonol accumulation in response to the cold (Zhao et al., 2019). Similarly, *Grain Size and Abiotic stress tolerance 1* (*GSA1*) in rice gene encoding for UGTs is required for the redirection of metabolic flux from lignin biosynthesis to flavonoid biosynthesis under heat stress and the accumulation of flavonoid glycosides, which protect rice against abiotic stress (Dong et al., 2020). In combination, these imply that UGTs are conserved and play crucial roles in response to low temperatures in various plant species by protecting plants against reactive oxygen species (ROS) or oxidative stress via the accumulation of flavonols. This means that these mechanisms may conserved among ancient tropical C_4 grasses.

Another commonly expressed gene category among the tropical C_4 grasses in this study is the genes involved in active ion transmembrane transporters. One of the most important active ion transmembrane transporters involved in responding to low temperatures in several plants is the Ca^{2+} channel. At low temperatures, Ca^{2+} plays a role as a messenger in a low-temperature signal transduction pathway. In rice (*Oryza sativa*) (Ma et al., 2015), *A. thaliana* (Knight et al., 1996; Polisensky et al., 1996) and alfalfa (Monroy et al., 1995), influxes of Ca^{2+} from extracellular stores to cytoplasm occur in responding to low temperatures. In *A. thaliana*, the Ca^{2+} is related to an increased expression of several cold-induced genes including the CRT/DRE-controlled COR6 and KIN1 genes (Monroy et al., 1993, 1995; Knight et al., 1996). As there is no clear study of cytosolic Ca^{2+} influx in the grass due to the cold and its downstream mechanisms, our study suggests that the tropical C_4 grasses may use this mechanism to respond to the cold.

Another important transporter responding to low temperatures is the plasma membrane H+-ATPase, a proton pump that plays a central role in physiological functions such as intracellular pH regulation, stomatal opening, nutrient uptake, and cell growth (Janicka-Russak, 2011). There are several studies on the H+-ATPase at low temperatures in C_3 grass species. For example, in winter wheat, the H+-ATPase was increased in seedlings at 5 ºC as they hardened (Jian et al., 1982). Additionally, the H+-ATPase activity in chilling-insensitive rice (*Oryza sativa*) increased at low

temperatures, whereas it was slightly dropped in chilling-sensitive plants (Kasamo, 1988). Although there is no study on this transporter in C_4 grasses, our study together with other studies in grasses has implied that the C_4 tropical grasses may commonly respond to low temperatures by altering H+-ATPase activity.

General mechanisms responding to low temperatures in the C_3 temperate grass

Our study reveals species-specific responses to low temperatures, particularly evident in the C_3 temperate grass, BSY. Gene expression analysis highlights an upregulation of pathways associated with ROS metabolism and response, as well as the generation of precursor metabolites and energy in response to cold stress. This suggests that BSY employs mechanisms to prevent photodamage from reactive oxygen species (ROS), potentially through ROS scavenging or antioxidant activities. Additionally, the increased metabolic activity indicates a rising demand for energy to sustain cellular processes under low-temperature conditions.

Moreover, we used *A. thaliana*, which is a dicot angiosperm, as a reference for gene annotation. This indicates that this gene group may be conserved across several monocot and dicot plants. To confirm this implication, further studies are needed.

Common cold-responsive mechanisms in both temperate C_3 and tropical C_4 grasses

Our study shows that 12 of 17 genes that are commonly expressed among both C_3 temperate and C_4 tropical grasses show the same expression patterns among all species; eight genes were downregulated, while the others were upregulated. This implies that these genes may be involved in commonly conserved mechanisms responding to low temperatures in the grass family.

Closely looking into the gene annotation and functions of eight down-regulated genes in all three species (Table S4.4), there are three genes, which are DIOX2, SPS1, and VDE, that are involved in responding to photooxidative stress via reactive oxygen species (ROS) scavenging. Each gene is involved in different biosynthesis of antioxidants, which are coumarin scopoletin (Döll et al., 2018), plastoquinone-9 (PQ-

9) (Hirooka et al., 2003; Block et al., 2013; Ksas et al., 2015), and zeaxanthin (Niyogi et al., 1998; Havaux et al., 2000; Hieber et al., 2002), respectively. Thus, downregulation of these three genes may result in a lower abundance of ROS scavenging molecules and reduced resistance to photooxidative stress leading to an increase in photosystem II (PSII) photoinhibition and a reduction in PSII photosynthetic efficiency. The other two down-regulated genes, SPA4 and IAA26, are related to plant growth and development. The downregulation of SPA4 induces photomorphogenesis in the phytochrome signalling pathway (Laubinger et al., 2003, 2006; Fittinghoff et al., 2006), which may result in more plant growth and development. Additionally, the low expressed IAA26 may influence the drop in an auxin transcription factor, which acts as a repressor of early auxin response genes, leading to less expression of the auxin gene followed by slow or inhibited plant growth and development (Liscum et al., 2002). Our result also shows that the expression of INO2 is decreased affecting the reduction of myo-inositol biosynthesis. Myo-inositol is a crucial metabolite acting as a central feature in plant biochemistry and physiology involved in various mechanisms such as cell wall and membrane biogenesis, stress responses and the regulation of cell death, auxin physiology, and osmoregulation (Loewus et al., 2000; Eckardt, 2010). Thus, the decrease in myo-inositol due to low temperature may lead to deterioration or dysfunctions in various mechanisms as mentioned before. The other two genes showing downregulation across the three species are TRP5 and SVR3, which are transcription regulators. TRP5 encodes a telomerase repeat-binding protein 5 located in the nucleus. The reduction in this protein may lead to DNA damage at telomeres and inhibit telomere replication (Kusová et al., 2023). SVR3, which is encoded for a chloroplastic elongation factor required for proper rRNA processing in *A. thaliana* at low temperatures (Liu et al., 2010), is downregulated in all three species with similar levels of gene expression (log2fold change between -1.14 and -1.39) (Figure 4.9 and Supplement: Table S4.4). The downregulation of SVR3 may lead to the reduction in chloroplastic rRNA resulting in the decrease of gene expression in chloroplast that may be involved in various processes such as photosynthesis, the repairment of photosystem due to photodamage, and so on.

Among the four genes that are upregulated in all three species, CADH9 exhibits high expression levels and is associated with increased lignin biosynthesis, a crucial

component of the secondary cell wall (Eudes et al., 2006; Liu et al., 2018). This suggests that both C_3 temperate and C_4 tropical species in this study may respond to chilling temperatures by accumulating more lignin, enhancing cell wall thickness and rigidity, and potentially protecting plants from cellular damage (Liu et al., 2018) CXE3, which encodes Carboxyesterase 3 activity, plays a pivotal role in hydrolysing diverse ester compounds within plants. This enzymatic activity is significant in influencing multiple aspects of plant biology, including growth, development, stress responses, and the activation of herbicidal substances (Wang et al., 2022). Despite its established functions, there remains a gap in the scientific literature regarding the response of CXE3 to low-temperature conditions. The next gene, FLZ18, encodes FCS-Like Zinc finger 18 protein, which facilitates SnRK1 (SNF1-related protein kinase 1) activity. It downregulates ATP-consuming biosynthetic processes while upregulating the energy-generating catabolism (Börnke, 2014). This suggests that plants tend to reduce ATP usage and obtain more energy from alternative pathways during growth at low temperatures. Interestingly, there is one novel gene that is highly expressed in all three species, especially in both C_4 tropical species which is about 2.1 to 2.7 times higher than the C_3 temperate (Figure 4.9 and Supplement: Table S4.4) This novel gene cannot be annotated using *A. thaliana* as a reference database. Additionally, it is matched with the sequence of predicted ternary complex factor MIP1 (Movement Protein-Interacting Protein 1) -like protein of maize (*Zea mays*) in the NCBI database (Accession no.: XM_035965421.1 and XM_008672029.3) with high confidence (82.47% identity and E-value < 0). Further studies are required to unravel its functions and underlying mechanisms.

Focusing on the genes that are upregulated only in both C_4 tropical species but downregulated in the C_3 temperate, there are two genes showing this pattern. The first gene, PTR2, Peptide Transporters 2, plays a role in the transport of diverse types of peptides (Choi et al., 2020). The second gene, MSSP2, encodes a monosaccharide-sensing protein 2 that is involved in the transport of sugars to the tonoplast or vacuole (Wormit et al., 2006; Schulz et al., 2011). Taken together, these findings suggest that C_4 tropical species respond to low temperatures by enhancing the transport of peptides and monosaccharides, which are subsequently stored in the vacuole rather than being

loaded into the phloem. In contrast, the C_3 temperate species may exhibit an opposing response or potentially use alternative mechanisms.

Furthermore, there are two genes, MENB and PAC, that exhibit an upregulation pattern in C_3 species, whereas they are downregulated in both C_4 species. The MENB gene is primarily involved in the biosynthesis of phylloquinone, also known as vitamin K1, which plays a crucial role in electron transfer within photosystem I (PSI) (Oostende et al., 2008). This suggests that the C_3 temperate species uses this mechanism to maintain the function of PSI and enhance photosynthetic efficiency under low-temperature conditions. In contrast, both C_4 tropical species may utilise alternative mechanisms to sustain PSI function, or it is possible that their PSI function is compromised due to a deficiency in vitamin K1. For the PAC gene, this gene is involved in chloroplast differentiation from proplastids or etioplasts. Additionally, it potentially regulates the chloroplast-encoded gene expression and mRNA maturation (Reiter et al., 1994; Grevelding et al., 1996; Meurer et al., 1998). These findings suggest that the C_3 species maintain the chloroplast functions during growth under low-temperature conditions by upregulating the expression of this gene. Conversely, it is plausible that the chloroplast functions may be compromised in C_4 tropical species, or alternative mechanisms are employed in place of the PAC gene.

We conducted a thorough examination to identify conserved genes reported in previous studies. Our analysis revealed that some photosynthetic genes were absent in certain species, possibly due to the removal of transcripts encoding these genes owing to low sequence quality or RNA degradation prior to sequencing. Notably, we observed downregulation of PPDK in SCO, aligning with our expectation of decreased photosynthetic efficiency under cold conditions, a phenomenon documented in various grass species. For instance, in maize (*Zea mays*), a C_4 grass, mRNA transcripts for PPDK decreased by approximately 50% after exposure to 14 °C day/ 10 °C night temperatures for 14 days (Long et al., 2013).

Interestingly, we noted upregulation of RubisCo in CBR, a tropical C_4 species. This finding suggests that some tropical C_4 grasses, like CBR, may possess mechanisms to sustain photosynthetic efficiency at low temperatures. However, this hypothesis warrants validation through RubisCo activity measurement and/or physiological assessments.

Furthermore, most putative cold-responsive genes were present in all three species with high similarity levels (77-88%). While putative CBF genes (CBF1, CBF2, CBF3, and CBF4) were identified in the C_3 temperate grass, BSY, only a subset was found in the C_4 tropical species. This discrepancy suggests that CBF expression may be common in C_3 but not all C_4 species. Additionally, our results indicated the presence of the putative WCS19 gene in both C_4 species, implying conservation within C_4 grasses. Conversely, the TaIRIP gene was exclusively detected in BSY, suggesting conservation only in C_3 temperate grasses, requiring further validation. Notably, there is a limited number of transcripts in each de novo transcriptome compared to the total number of genes in each species.

6. Conclusions

In this study, we investigated the transcriptional responses of three grass species belonging to distantly related subfamilies to short-term chilling temperatures. The primary objective was to explore whether C_3 temperate Pooideae grasses share ancestral cold-tolerance mechanisms with C_4 grasses. To address this question, we compared the differentially expressed genes (DEGs) and examined their functional implications between BSY (a C_3 temperate Pooideae grass) and two C_4 tropical grasses, namely CBR from the subfamily Panicoideae and SCO from the subfamily Chloridoideae.

Furthermore, we explored whether genes in C_3 temperate species show common expression patterns observed in the C_4 response to low temperatures. The results indicate that C_3 temperate grasses share diverse common expression patterns with C_4 tropical species in several aspects, including the reduction of reactive oxygen species (ROS) scavenging, inducing of photoinhibition in photosystem II leading to decreased photosynthetic efficiency, inhibition of cellular and chloroplast gene expressions, as well as impacts on plant growth and development.

Although C_3 temperate grasses share some common gene expressions with C_4 tropical grasses in response to cold, they exhibit unique responses in certain aspects. Our results demonstrate that C_3 temperate grasses have developed mechanisms to maintain photosynthesis through phylloquinone (vitamin K1) biosynthesis and

increased chloroplast differentiation. In contrast, C_4 tropical grasses show increased peptide and sugar transport for storage in vacuoles, which is less prominent in C_3 grasses.

In conclusion, our study has shown that C_3 temperate Pooideae grasses have shared some gene expression patterns and utilise similar mechanisms in response to cold from the C_4 species. However, they have also developed distinct mechanisms to adapt and survive in low-temperature environments. It is important to note that these results are based on only three grass species. Therefore, further studies on additional species are needed to broaden these findings.

7. Acknowledgements

I would like to thank Dr. Teera Wacharamonkol, for conducting the chilling treatment and collecting leaf samples used in this study. I gratefully thank Dr. Matheus Bianconi for training me in plant biomolecular techniques This work was funded by a studentship awarded by the Royal Thai Government.

8. Supplementary data

Table S4.1 The number of raw reads from the two units of RNA-seq and cleaned reads after quality check and adapter trimming

Species	Treatment	Sample ID	Sequencing Direction	No. of reads			
				Unit 1		Unit 2	
				Raw	Cleaned	Raw	Cleaned
BSY	Control	BSY_C1	Forward	5983895	4870387	6342683	5258365
		BSY_C1	Reverse	6052369	4924712	6641750	5276894
		BSY_C2	Forward	6818729	6132657	6763171	6184885
		BSY_C2	Reverse	6681019	6029145	6930680	6028578
		BSY_C4	Forward	5915733	4864331	5898910	4902896
		BSY_C4	Reverse	6030063	4927209	6249185	4985172
	Chilling	BSY_T1	Forward	4427326	3852211	5199343	4654550
		BSY_T1	Reverse	4353105	3846060	5426809	4610938
		BSY_T2	Forward	5426049	4516319	5308123	4470754
		BSY_T2	Reverse	5544853	4594510	5666365	4622282
		BSY_T4	Forward	5955570	5129252	6047314	5285894
		BSY_T4	Reverse	6004193	5129309	6359303	5273980
CBR	Control	CBR_C1	Forward	8368069	7482598	7982115	7365833
		CBR_C1	Reverse	8467712	7629586	8321683	7409228
		CBR_C2	Forward	6422365	5758847	6626457	6134212
		CBR_C2	Reverse	6585941	5880444	7014900	6196717
		CBR_C4	Forward	6020716	4966536	6567075	5353171
		CBR_C4	Reverse	6262847	4933066	7014907	5318577
	Chilling	CBR_T1	Forward	7011153	6225255	7110703	6491167
		CBR_T1	Reverse	7080565	6267094	7453137	6523700
		CBR_T2	Forward	5269905	4406321	5193408	4434037
		CBR_T2	Reverse	5275188	4354730	5508419	4476932
		CBR_T4	Forward	3024810	2459747	3191573	2601455
		CBR_T4	Reverse	3089507	2442376	3429603	2614608
SCO	Control	SCO_C1	Forward	1863608	916374	1792010	989836
		SCO_C1	Reverse	1969215	944671	2022633	1057792
		SCO_C2	Forward	2961996	2262087	3169228	2374685
		SCO_C2	Reverse	3290658	2271000	3509447	2358684
		SCO_C4	Forward	5395862	4516585	5499859	4690125
		SCO_C4	Reverse	5454023	4556775	5826842	4839972
	Chilling	SCO_T1	Forward	1274260	652920	1221577	693488
		SCO_T1	Reverse	1340316	662301	1369863	752896
		SCO_T2	Forward	3346931	2494345	3560812	2572379
		SCO_T2	Reverse	3595368	285117	3921732	2616075
		SCO_T4	Forward	1392193	943096	1473019	1019051
		SCO_T4	Reverse	1525628	954258	1646941	1041953

Table S4.2 the details of RNA samples before and after quality checking (QC) before performing RNA library preparations.

Species	Treat.	Sample ID	Before QC		After QC			
			Sample Conc. (ng/ul)	Volume (ul)	QC Conc. (ng/ul)	Volume (ul)	Total mass (ng)	RIN Values
BSY	Control	BSY_C1	36.12	30	9.55	32	305.62	3.5
		BSY_C2	34.9	30	5.41	30	162.44	4.4
		BSY_C4	33.93	30	8.72	32	278.95	2.4
	Chilling	BSY _T1	26.47	30	8.91	32	285.03	2.0
		BSY_T2	44.075	30	4.71	32	150.62	3.0
		BSY_T4	37.44	30	8.39	30	251.68	6.7
CBR	Control	CBR_C1	30.48	30	24.63	32	788.23	2.7
		CBR_C2	36.58	30	17.44	32	558.16	3.3
		CBR_C4	32.21	30	24.66	32	789.08	1.5
	Chilling	CBR_T1	32.1	30	22.15	32	708.95	2.0
		CBR_T2	37	30	26.10	32	835.13	1.2
		CBR_T4	30.82	30	21.99	32	703.52	1.7
SCO	Control	SCO_C1	28.615	30	4.98	32	159.23	3.2
		SCO_C2	33.16	30	8.58	32	274.55	2.5
		SCO_C4	42.49	30	19.70	32	630.33	2.4
	Chilling	SCO_T1	30.76	30	13.05	32	417.66	1.5
		SCO_T2	32.69	30	5.25	30	157.61	0.0
		SCO_T4	45.43	30	17.71	31	549.15	0.0

Note: Abbrev.: Species: *Cenchrus brownii* (CBR), *Sporobolus consimilis* (SCO), and *Beckmannia syzigachne* (BSY). Treat. means the temperature treatments in this study. RIN is RNA integrity number ranging from 10 to 0. The lower RIN, the RNA quality.

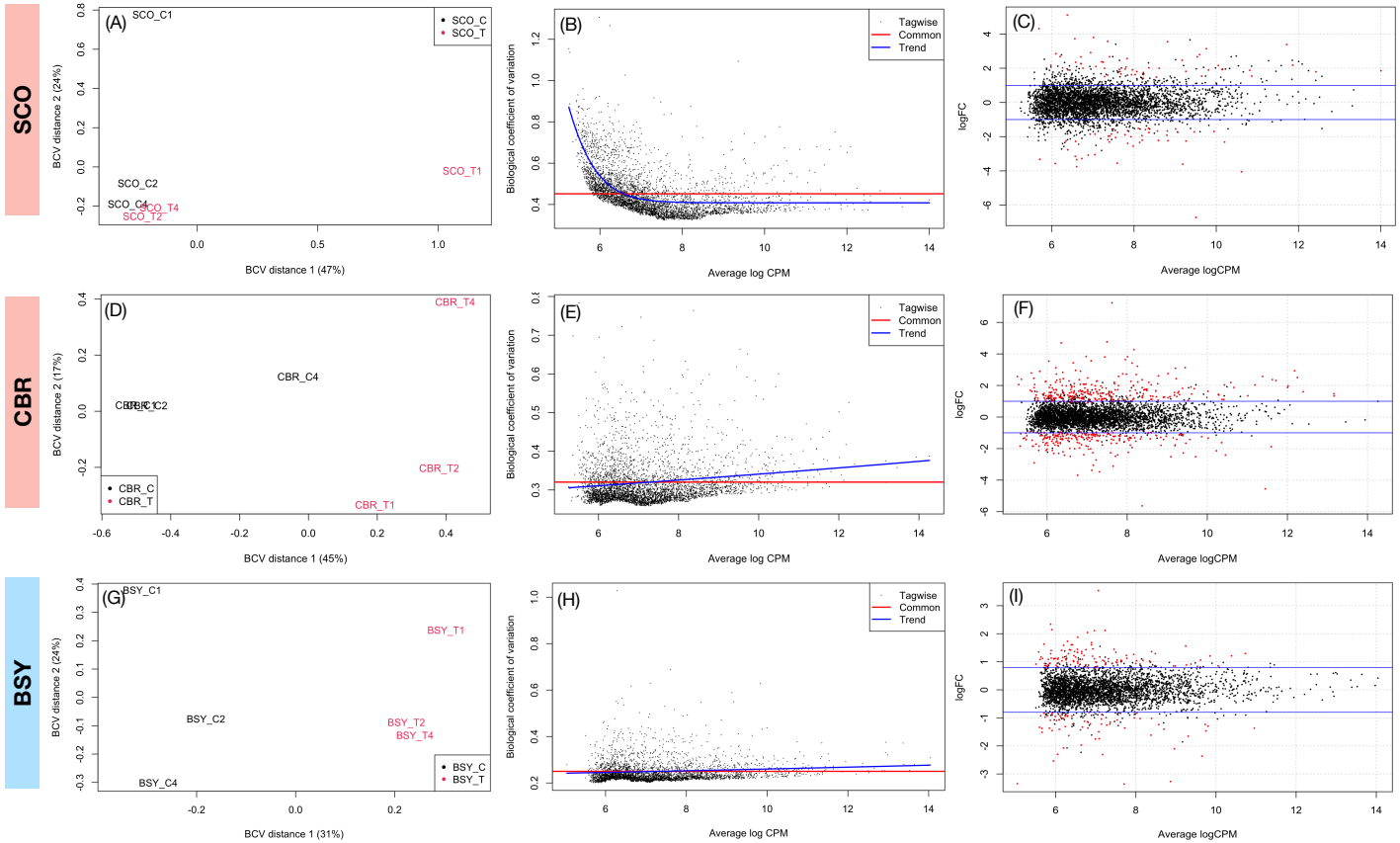

Figure S4.1 Differential expression genes (DEGs) analysis. Multidimensional scaling plot (A, D, and G) shows distances between gene expression profiles approximate the typical log2 fold changes between the samples; black is the control, while red is the chilling treatment. Plot biological coefficient of variation (BCV) against gene abundance (in average log2 counts per million (CPM)) (B, E, and H) shows the dispersion of gene expression (blue) and the dispersion estimated by generalised linear model (red). Smear plots (C, F, and I) show the significant differences in DEGs (red dots).

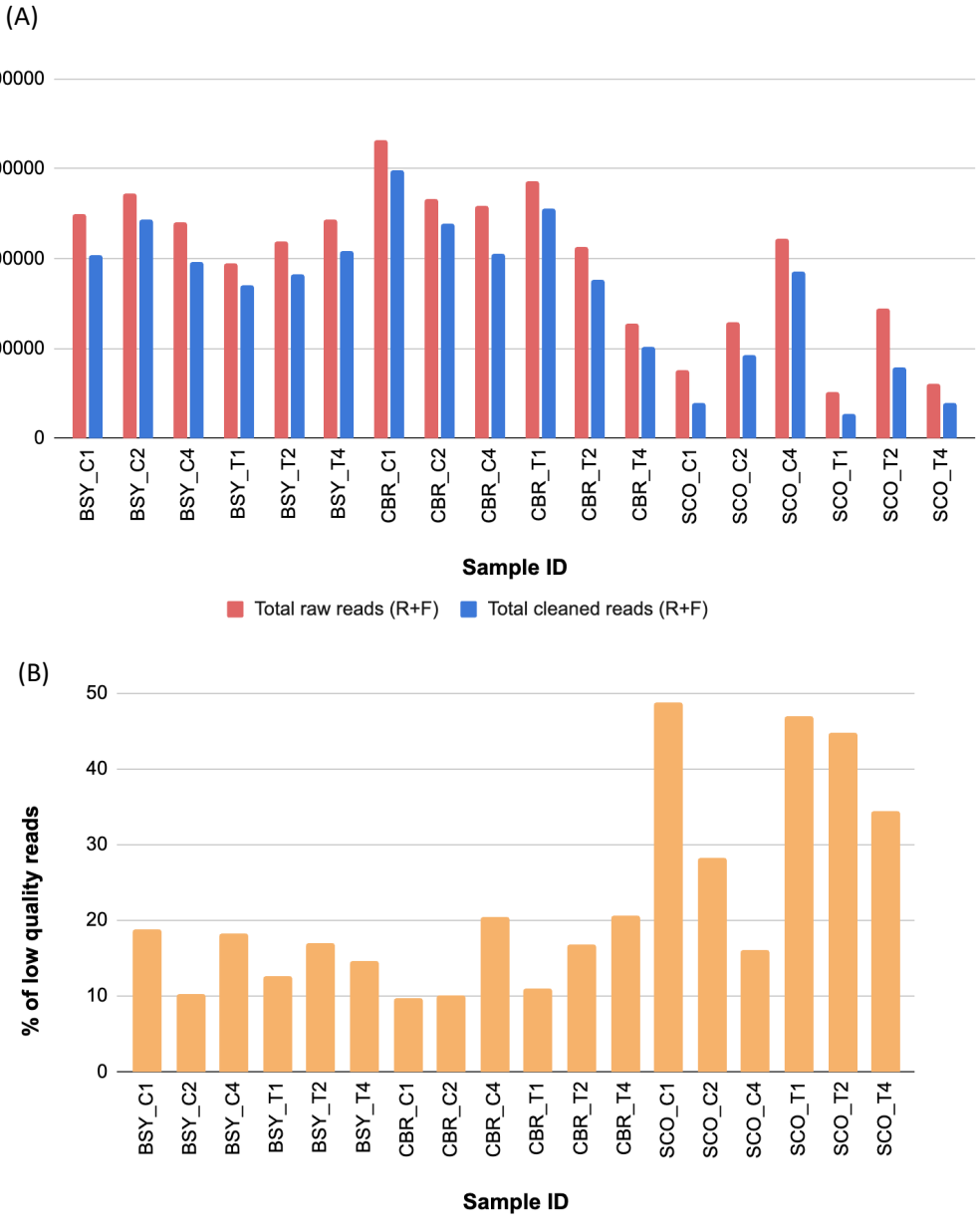

Figure S4.2 RNA sequencing and the quality check (QC) of raw reads of each sample in this study by FastQC. (A) shows the total number of raw reads (red) compared to the number of cleaned reads (blue) after QC. (B) shows the percentage of low-quality reads that were removed before *De novo* transcriptomic assembly. Sample ID is named by 'SpeciesName_TreatmentReplicate'. The treatments are control (C) and chilling (T). The abbreviations of each species name are *Beckmannia syzigachne* (BSY), *Cenchrus brownii* (CBR), and *Sporobolus consimilis* (SCO)

Table S4.3 The number of transcripts after assembly by Trinity and gene annotation

Species	Total number of transcripts		
	Assembles	**Annotated**	**Unannotated**
SCO	286,719	109,066	177,653
CBR	551,972	157,184	394,788
BSY	320,149	89,220	230,929

Table S4.4 The expression level, annotation, and function(s) of the 17 co-expressed genes among two C_4 tropical and a C_3 temperate grasses in this study.

Gene ID	Log2 fold change			UniProt Name	UniProt Entry	Gene Name/ Synonym	Annotation/Functions
	BSY	CBR	SCO				
1.	-0.619	-2.515	-3.588	INO2	Q38862	IPS2, MIPS2, At2g22240, T26C19.10	- Inositol-3-phosphate synthase isozyme 2 - The key enzyme involved in the biosynthesis pathway of myo-inositol converts glucose 6-phosphate to 1-myo-inositol 1-phosphate in an NAD-dependent manner. MIPS2 and MIPS3 are predominantly found in vascular or related tissues and are located within the cytoplasm (Donahue et al., 2010). Myo-inositol plays a critical role as a cellular metabolite, serving as the foundational structure for various lipid signalling molecules that participate in multiple pathways, including stress responses, cell death regulation, auxin perception, cell wall biosynthesis, and ascorbic acid synthesis (Eckardt, 2010).
2.	-1.392	-1.508	-1.922	DIOX2	JF681954.1 Q84MB6	At3g50210, F11C1.50	- Probable a 2-oxoglutarate-dependent dioxygenase - Involved in the production of a simple coumarin called scopoletin, which serves as a secondary metabolite in plants, aiding in their adaptation to the environment and providing defence against plant pathogens (Chong et al., 2002; Carpinella et al., 2005; Sun et al., 2015). Low-temperature stress can trigger the accumulation of coumarin scopolin in leaves. Its role in leaves is believed to involve both antibiosis, due to the phenolic component's toxicity, and scavenging of reactive oxygen species (ROS) (Döll et al., 2018).

Gene ID	Log2 fold change			UniProt Name	UniProt Entry	Gene Name/ Synonym	Annotation/Functions
	BSY	CBR	SCO				
3.	-0.664	-1.930	-1.771	SPA4	Q94BM7	SPA4, At1g53090, F8L10.5	- Protein SPA1-RELATED 4 - This gene acts as a suppressor of photomorphogenesis when exposed to light. Probably a component of the COP1/SPA E3 ubiquitin-protein ligase complex, as suggested by (Laubinger et al., 2003, 2006; Fittinghoff et al., 2006).
4.	-1.069	-1.145	-1.522	SPS1	Q8S948	SPS1, SPPS, SPS, At1g78510, T30F21.15	- Solanesyl diphosphate synthase 1, chloroplastic - Involved in providing solanesyl diphosphate for plastoquinone-9 (PQ-9) formation in plastids (Hirooka et al., 2003; Block et al., 2013; Ksas et al., 2015). PQ-9 is a lipophilic antioxidant that acts as protectant against photooxidative stress under high light stress conditions (Block et al., 2013; Ksas et al., 2015; Ferretti et al., 2018). - In *Oryza sativa subsp. japonica* (Uniprot Entry Q653T6): Involved in the supply of solanesyl diphosphate for ubiquinone-9 (UQ-9) biosynthesis in mitochondria, which has a different mechanism for the supply of isoprenoid precursors in UQ biosynthesis from *Arabidopsis thaliana*, in which SPS1 provides a prenyl moiety for UQ9 at the endoplasmic reticulum (Ohara et al., 2010).
5.	-0.806	-1.328	-1.294	VDE	Q39249	VDE1, AVDE1, NPQ1, VXDE, At1g08550, F22O13.3, T27G7.23	- Violaxanthin de-epoxidase, chloroplastic - A component of the xanthophyll cycle, controlling zeaxanthin levels in chloroplasts. Zeaxanthin triggers the dissipation of excitation energy within the chlorophyll of the photosystem II light-harvesting protein complex (Niyogi et al., 1998; Havaux et al., 2000; Hieber et al., 2002)

Gene ID	Log2 fold change			UniProt Name	UniProt Entry	Gene Name/ Synonym	Annotation/Functions
	BSY	CBR	SCO				
6.	-0.534	-1.209	-1.179	TRP5	Q6R0E3	TRP5, TRFL2, At1g07540, F22G5.8	- Telomerase repeat-binding protein 5 - It specifically attaches to the double-stranded DNA sequences found in plant telomeres. A minimum of 6 repeats of these telomeric sequences is necessary for the binding process.
7.	-1.391	-1.381	-1.144	SVR3	F4K410	SVR3, At5g13650, MSH12.11	- Putative elongation factor TypA-like SVR3, chloroplastic - Putative chloroplastic elongation factor involved in response to chilling stress. Required for proper chloroplast rRNA processing and/or translation at low temperature and involved in plastid protein homeostasis (Saini et al., 2011)
8.	-1.263	-1.380	-0.939	IAA26	Q8LAL2	IAA26, PAP1, At3g16500, MDC8.13	- Auxin-responsive protein - Aux/IAA proteins, which are short-lived transcriptional factors, act as repressors of early auxin response genes when auxin concentrations are low. This repression is believed to occur through interactions with auxin response factors (ARFs), which bind to the auxin-responsive promoter element (AuxRE). The formation of heterodimers with ARF proteins could change their ability to regulate the expression of early auxin response genes(Liscum et al., 2002). - In *Oryza sativa subsp. japonica* (UniProt Entry Q652A1)
9.	0.914	1.158	1.065	CXE3	Q9FX92	CXE3, At1g49640, F14J22.12	- Probable carboxylesterase 3 - Carboxylesterase acting on esters with varying acyl chain length.

Gene ID	Log2 fold change			UniProt Name	UniProt Entry	Gene Name/ Synonym	Annotation/Functions
	BSY	CBR	SCO				
10.	0.847	2.190	1.647	CADH9	P42734	CAD9, CAD1, At4g39330, T22F8.230	- Probable cinnamyl alcohol dehydrogenase 9 - Involved in lignin biosynthesis (Eudes et al., 2006). - In *Oryza sativa subsp. japonica* (UniProt Entry Q10PS6): Involved in lignin biosynthesis.
11.	1.408	1.742	2.033	FLZ18	P0DO12	FLZ18, DUF581-4, At1g53903, T28A20	- FCS-Like Zinc finger 18 - It could serve as a mediator to enable the SnRK1 complex to interact with effector proteins, leading to tissue- and stimulus-specific variations in the SnRK1 regulatory pathway. SNF1-related kinase (SnRK1) adjusts to carbohydrate availability and environmental stresses by suppressing ATP-consuming biosynthesis processes while promoting energy-generating catabolic reactions through gene expression and post-transcriptional regulation. (Börnke, 2014)
12.	0.766	1.633	2.096	*NA*	*NA*	NA	NA
13.	-0.519	0.749	1.077	PTR2	P46032	NPF8.3, NTR1, PTR2, PTR2-B, At2g02040, F14H20.11	- Protein NRT1/ PTR FAMILY 8.3 - Peptide transporter. Mediates the transport of di- and tripeptides. High affinity, low-capacity transporter. Can also transport histidine.
14.	-0.743	1.033	1.248	MSSP2	Q8LPQ8	MSSP2, TMT2, At4g35300,	- Monosaccharide-sensing protein 2 (or Tonoplast monosaccharide transporter 2 (TMT2)) - Sugar proton-coupled antiporter which contributes to vacuolar

Gene ID	Log2 fold change			UniProt Name	UniProt Entry	Gene Name/ Synonym	Annotation/Functions
	BSY	CBR	SCO				
						F23E12.140	sugar import (e.g. monosaccharides including glucose, sucrose and fructose), particularly during stress responses (e.g. in response to cold) (Wormit et al., 2006; Schulz et al., 2011).
15.	0.653	-1.297	-1.328	MENB	Q8GYN9	MENB, At1g60550, F8A5.9	- 1,4-dihydroxy-2-naphthoyl-CoA synthase, peroxisomal - Involved in the biosynthesis of phylloquinone (vitamin K1). It is responsible for the one electron transfer at the A_1 site of photosystem I, a process that involves turnover between the quinone and semi-quinone forms of phylloquinone (Oostende et al., 2008).
16.	0.968	-1.068	-1.207	PAC	Q39089	PAC, t9j23, At2g48120, F11L15.2	- Protein PALE CRESS, chloroplastic - Required for the differentiation of chloroplast from proplastids or etioplasts, probably by modulating some chloroplast-encoded genes expression and mRNA maturation. Involved in leaf-cells differentiation (Reiter et al., 1994; Grevelding et al., 1996; Meurer et al., 1998).
17.	-1.054	-1.092	0.819	ANTR1	O82390	ANTR1, PHT4;1, At2g29650, T27A16.25	- Sodium-dependent phosphate transport protein 1, chloroplastic - Specific for inorganic phosphate transport across the thylakoid membrane in a sodium dependent manner. Binds glutamate but cannot transport it. May act as an ascorbate transporter at the thylakoid membrane (Probable) (Guo et al., 2008; Pavón et al., 2008; Miyaji et al., 2015).

Note: UniProt name and entry, gene annotation and functions are based on the UniProt public database of *Arabidopsis thaliana*. 'NA' means the gene annotation and function are not available on public databases.

Table S4.5 The results of searching for photosynthetic-involved genes and cold-responsive genes that have been reported in closely related species by using BlastN.

Gene name	Species	Accession no.	Sequence ID	Identity (%)	Length (bp)	E-value	Bit score	logFC	logCPM	p-value
PEPC (Phosphoenolpyruvate carboxylase)										
PEPC	*Arabidopsis thaliana*	AF071788.1	BSY_DN15090_c0_g1_i1	76.151	2650	0	1352	-0.446	7.204	0.154
PEPC	*Arabidopsis thaliana*	AF071788.1	CBR_DN1211_c1_g1_i4	76.713	2087	0	1140	NA	NA	NA
PEPC	*Arabidopsis thaliana*	AF071788.1	SCO_DN2617_c0_g1_i3	75.316	2844	0	1332	NA	NA	NA
PPDK (pyruvate orthophosphate dikinase)										
PPDK	*Zea mays*	AH004068.2	BSY_DN2915_c0_g1_i2	90.254	236	2.13E-82	309	0.013	10.695	0.961
PPDK	*Zea mays*	AH004068.2	CBR_DN6446_c0_g1_i5	86.975	238	7.99E-69	265	-0.308	13.457	0.434
PPDK	*Zea mays*	AH004068.2	SCO_DN9477_c0_g1_i5	83.691	233	8.43E-56	220	NA	NA	NA
RubisCo (Ribulose 1,5-bisphosphate carboxylase/oxygenase)										
RubisCo	*Oryza sativa*	L24073.1	BSY_DN458_c0_g1_i26	95.215	1045	0	1653	NA	NA	NA
RubisCo	*Oryza sativa*	L24073.1	CBR_DN11474_c0_g1_i12	95.694	1045	0	1681	0.644	7.236	0.214
RubisCo	*Oryza sativa*	L24073.1	SCO_DN49687_c0_g1_i12	95.311	1045	0	1659	NA	NA	NA
RubisCo	*Triticum aestivum*	LT576864.1	BSY_DN458_c0_g1_i26	97.839	1388	0	2398	NA	NA	NA
RubisCo	*Triticum aestivum*	LT576864.1	CBR_DN11474_c0_g1_i12	96.637	1368	0	2270	0.644	7.236	0.214
RubisCo	*Triticum aestivum*	LT576864.1	SCO_DN49687_c0_g1_i12	95.968	1389	0	2255	NA	NA	NA
CBF (C-repeat/DRE binding factor)										
CBF1	*Triticum aestivum*	EF028751.1	BSY_DN7490_c0_g1_i2	84.72	733	0	710	NA	NA	NA
CBF1	*Triticum aestivum*	EF028751.1	CBR_DN30264_c0_g2_i2	82.456	627	1.88E-143	512	NA	NA	NA
CBF2	*Triticum aestivum*	EF028752.1	BSY_DN90006_c0_g1_i1	81.404	570	5.90E-122	440	NA	NA	NA
CBF2	*Triticum aestivum*	EF028752.1	CBR_DN6116_c4_g1_i1	87.619	210	3.04E-62	243	NA	NA	NA
CBF2	*Triticum aestivum*	EF028752.1	SCO_DN24841_c0_g1_i1	87.984	258	1.84E-81	305	NA	NA	NA

Gene name	Species	Accession no.	Sequence ID	Identity (%)	Length (bp)	E-value	Bit score	logFC	logCPM	p-value
CBF (C-repeat/DRE binding factor) [cont.]										
CBF3	*Lolium perenne*	AY960831.1	BSY_DN2901_c0_g1_i4	79.365	756	1.84E-13	472	NA	NA	NA
CBF3	*Lolium perenne*	AY960831.1	SCO_DN81169_c0_g1_i1	82.707	266	1.29E-57	226	NA	NA	NA
CBF3	*Triticum aestivum*	EF028755.1	BSY_DN2901_c0_g1_i8	81.107	831	2.95E-17	597	NA	NA	NA
CBF3	*Triticum aestivum*	EF028755.1	CBR_DN1776_c0_g1_i21	78.858	648	5.48E-11	398	NA	NA	NA
CBF3	*Triticum aestivum*	EF028755.1	SCO_DN34668_c0_g1_i1	77.823	487	2.15E-70	268	NA	NA	NA
CBF4	*Triticum aestivum*	EF028769.1	BSY_DN2901_c0_g1_i19	84.874	357	7.00E-96	353	NA	NA	NA
CBF4	*Triticum aestivum*	EF028769.1	SCO_DN991_c1_g1_i6	77.617	277	1.34E-37	159	NA	NA	NA
COR (cold-regulated proteins)										
WCOR14	*Triticum aestivum*	FJ655858.1	BSY_DN616_c0_g1_i1	84.472	161	1.89E-34	148	NA	NA	NA
WCOR14	*Triticum aestivum*	FJ655858.1	CBR_DN34379_c0_g1_i4	81.988	161	9.05E-30	134	NA	NA	NA
WCOR14	*Triticum aestivum*	FJ655858.1	SCO_DN16951_c0_g1_i1	81.988	161	4.35E-30	134	NA	NA	NA
WCS19	*Triticum aestivum*	L13437.2	CBR_DN34379_c0_g1_i4	80.6	433	2.32E-81	305	NA	NA	NA
WCS19	*Triticum aestivum*	L13437.2	SCO_DN16951_c0_g1_i1	80.139	433	2.40E-78	294	NA	NA	NA
DHN (Dehydrin)										
DHN5	*Hordeum vulgare*	AF181455.1	BSY_DN7609_c1_g3_i1	83.186	226	2.76E-50	204	NA	NA	NA
DHN5	*Hordeum vulgare*	AF181455.1	BSY_DN7609_c1_g3_i1	82.403	233	1.66E-47	195	NA	NA	NA
DHN5	*Hordeum vulgare*	AF181455.1	SCO_DN30219_c0_g1_i1	85.455	110	1.41E-22	111	NA	NA	NA
DHN8	*Hordeum vulgare*	AF043093.1	BSY_DN940_c0_g1_i2	84.914	643	3.24E-18	625	-2.404	9.852	1.60E-09
DHN8	*Hordeum vulgare*	AF043093.1	CBR_DN8349_c0_g1_i5	80.251	319	1.09E-54	219	-1.059	8.987	0.048
DHN8	*Hordeum vulgare*	AF043093.1	SCO_DN950_c0_g1_i4	88.260	477	9.80E-16	556	NA	NA	NA
DHN13	*Hordeum vulgare*	AY681974.1	BSY_DN71279_c0_g1_i4	82.243	535	5.68E-12	418	NA	NA	NA
DHN13	*Hordeum vulgare*	AY681974.1	CBR_DN39014_c0_g1_i1	82.151	437	2.79E-90	337	NA	NA	NA
DHN13	*Hordeum vulgare*	AY681974.1	SCO_DN1153_c0_g1_i14	82.773	476	1.01E-11	390	NA	NA	NA

Gene name	Species	Accession no.	Sequence ID	Identity (%)	Length (bp)	E-value	Bit score	logFC	logCPM	p-value
DHN (Dehydrin) [cont.]										
WCS120	*Triticum aestivum*	M93342.2	BSY_DN13032_c2_g1_i1	81.703	317	5.39E-69	265	NA	NA	NA
WCS120	*Triticum aestivum*	M93342.2	CBR_DN8349_c0_g1_i5	92.157	51	5.98E-11	73.1	-1.059	8.987	0.048
WCS120	*Triticum aestivum*	M93342.2	SCO_DN99857_c0_g2_i1	96.296	54	1.02E-15	87.9	NA	NA	NA
IRIP (Ice recrystallisation inhibition proteins)										
TaIRIP_1	*Triticum aestivum*	BE490254.1	BSY_DN12961_c0_g2_i1	85.310	565	8.59E-16	575	NA	NA	NA
TaIRIP_2	*Triticum aestivum*	BJ224369.1	BSY_DN12961_c0_g2_i1	84.340	811	0	784	NA	NA	NA
TaIRIP_3	*Triticum aestivum*	BE490074.1	BSY_DN12961_c0_g2_i1	85.739	589	1.06E-18	621	NA	NA	NA
FT (Fructosyl transferases)										
WFT1	*Triticum aestivum*	AB029887.1	BSY_DN1762_c0_g1_i17	79.952	1671	0	1199	-0.571	8.714	0.211
WFT1	*Triticum aestivum*	AB029887.1	CBR_DN155720_c0_g1_i1	82.724	492	1.27E-12	431	NA	NA	NA
WFT1	*Triticum aestivum*	AB029887.1	SCO_DN82304_c0_g1_i1	84.225	355	8.17E-93	344	NA	NA	NA
WFT2	*Triticum aestivum*	AB029888.1	BSY_DN1762_c0_g1_i6	79.768	1982	0	1378	NA	NA	NA
WFT2	*Triticum aestivum*	AB029888.1	CBR_DN352_c0_g1_i4	75.718	1742	0	802	-3.471	7.310	7.50E-07
WFT2	*Triticum aestivum*	AB029888.1	SCO_DN75925_c0_g1_i1	83.392	283	3.20E-67	259	NA	NA	NA
PpFT1	*Phleum pratense*	AB436697.1	BSY_DN1762_c0_g1_i15	88.068	2179	0	2542	NA	NA	NA
PpFT1	*Phleum pratense*	AB436697.1	CBR_DN352_c0_g1_i4	75.944	927	5.74E-13	459	-3.471	7.310	7.50E-07
PpFT1	*Phleum pratense*	AB436697.1	SCO_DN82304_c0_g1_i1	94.215	363	4.42E-16	551	NA	NA	NA

Note: Sequence ID represents an ID of transcript of each particular species obtained from *De Novo* assembly by Trinity. Species abbreviation: *Cenchrus brownii* (CBR), *Sporobolus consimilis* (SCO), and *Beckmannia syzigachne* (BSY). logFC = log fold change and logCPM = log count per million. The p-value is calculated during DEG analysis. NA means results not available (not presented) in the results of DEG analysis.

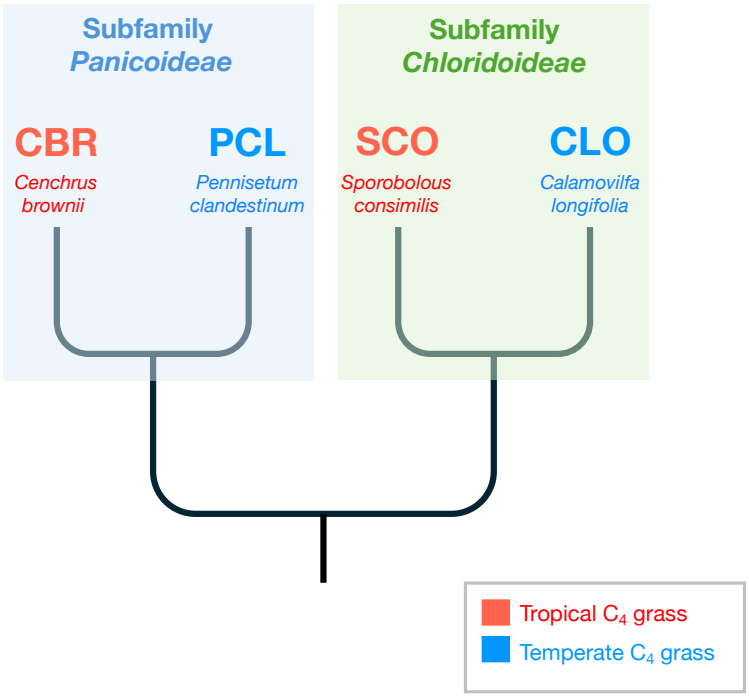

Figure S4.3 The phylogenetic tree showing the selected C_4 grasses from the original experimental plan

Chapter 5

Intraspecific physiological adaptations to short-term chilling temperatures at high-light intensity in *Alloteropsis semialata*

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Personal contribution:

I co-designed the study with Prof. Colin P. Osborne. Plant propagation and the chilling treatment were carried out by me. Finally, I performed all analyses, interpreted the results, and wrote the paper with the help of Prof. Colin P. Osborne.

1. Abstract

Low temperatures significantly influence plant growth and development by slowing cellular biochemical processes, notably photosynthesis. Despite lower activity, plants experience the same light energy levels, resulting in underutilisation and surplus energy. This excess energy causes photodamage within photosynthetic pathways, leading to photoinhibition and diminished photosynthetic efficiency. Our study explores intraspecific variation in photodamage induced by chilling and its association with photosynthetic pathways and geographic provenance. We hypothesise that environmental tolerance is more closely tied to geographic history than photosynthetic pathways, such that genotypes originating from colder climates are more tolerant of chilling. To investigate, we cultivated 12 genotypes of *A. semialata* with diverse geographical origins and photosynthetic types under high-light intensity with two phases of chilling treatment for 14 days. We measured the photosynthetic efficiency of photosystem II (PSII), non-photochemical quenching (NPQ), non-regulatory energy dissipation (NO), and leaf greenness, represented by the SPAD index, every two days and employed Phylogenetic Generalised Least Squares (PGLS) for analysis. Our findings indicate that all genotypes experienced negative effects from chilling-high light conditions to varying degrees. While PSII efficiency and NO decreased uniformly across all genotypes, NPQ increased differently across genotypes. The SPAD index exhibited variation among all genotypes. Moreover, physical changes and anthocyanin accumulation occurred in some genotypes. Interestingly, we observed a statistical relationship between the C_4 photosynthetic pathway and a decrease in both Phi2 and PhiNO, and an increase in PhiNPQ on day 14 after chilling, as well as a relationship between the non-C_4 pathway and a rising SPAD on day 8. These results suggest that the photosynthetic type rather than geographic origin may have a stronger influence over how *A. semialata* tolerates chilling in high-light environments.

2. Introduction

A major challenge for plants experiencing low temperatures is maintaining their photosynthetic efficiency. This is because biochemical processes are limited by the low activity and gene expression of photosynthetic enzymes such as RubisCo (Ribulose-1,5-bisphosphate carboxylase/oxygenase) and PEPC (Phosphoenolpyruvate carboxylase) (Long et al., 2013). These events result in a decrease in photosynthetic capacity, particularly in plants that are cold-sensitive or cannot acclimate to low temperatures (Long et al., 2013). Meanwhile, light is somewhat independent of temperature, which means that plants can simultaneously experience low temperatures and high light. The photons from light energy that can be used by photosynthesis are limited by low temperatures, which means that excess photons need to be dissipated via other mechanisms (i.e. quenched). If they cannot be quenched, these can trigger the production of reactive oxygen species (ROS) leading to damage of the reaction centre of photosystem II (PSII) and antenna pigments (Ruban, 2016). This phenomenon is called photodamage, which can inhibit photosynthesis (photoinhibition) and cause physical changes to plant leaves such as the yellowing of leaves (chlorosis) and cellular death (necrosis) (Wang et al., 2021) Thus, to maintain photosynthetic efficiency at low temperatures and high light intensity, plants need to have protective mechanisms to dissipate absorbed light energy.

Plants can release excess light energy through various mechanisms, including non-photochemical quenching (NPQ) and non-regulated processes (NO). NPQ describes a number of protective mechanisms that dissipate excess absorbed light energy as heat, which takes place in the photosynthetic membranes of plants (Ruban, 2016). NO processes refer to biochemical pathways or physiological responses that occur independent of regulatory mechanisms, such as hormonal signalling or environmental effects. These processes often serve essential functions in plant growth, development, and metabolism, however, they can produce by-products that can be harmful to plants or inhibit photosynthesis (Kuhlgert et al., 2016).

The alteration of leaf colour is a way plants respond to environmental stresses, serving as an indicator of either damage or protection. Regarding damage, plants unable to tolerate low temperatures may display yellowing of leaves, known as chlorosis. This

phenomenon arises from inhibited chlorophyll biosynthesis, resulting from suppressed gene expression and activity of biosynthetic enzymes, leading to reduced chlorophyll content in leaves (Zhao et al., 2020; Wang et al., 2021). Leaf chlorophyll content can be accessed via chemical or optical methods. In this study, we used an optical method, represented by the Special Products Analysis Division (SPAD) index, to measure relative leaf chlorophyll content, though not absolute content or concentration (Richardson et al., 2002; Uddling et al., 2007). Regarding to protective mechanisms against low temperatures, plant leaf colour may change depending on pigment accumulation. For instance, the accumulation of anthocyanin occurs in several grasses species including *Zoysia japonica* (Zoysiagrass) (Jin et al., 2022) growing under low temperatures and Rough bluegrass (*Poa trivialis L.*) under high-intensity light (Petrella et al., 2016). However, there is still a gap in studies examining anthocyanin accumulation under the combined influence of both conditions in grasses. This accumulation gives purple or red colour to leaves and it is involved in shielding the leaves against photoinhibition of PSII through screening of irradiance (Huner et al., 1998).

Alloteropsis semialata, commonly known as black seed or cockatoo grass, belongs to the Paniceae tribe within the subfamily Panicoideae. This species exhibits a range of photosynthetic types, encompassing C_3, C_3-C_4 intermediate, and C_4, along with a diverse geographical distribution characterised by varying levels of precipitation and temperature (Lundgren et al., 2016). This variation makes it an excellent model for exploring the evolutionary links between C_3 and C_4 traits and between these traits and environmental conditions.

How *A. semialata* adapts to lower temperatures remains relatively unexplored. Osborne *et al.* (2008) have shown that leaf growth and photosynthesis of the *A. semialata* C_3 and C_4 subspecies decline with temperature until 15 °C before being completely lost due to photoinhibition and photodamage. In addition, the C_3 subspecies can acclimate to chilling conditions after growing under 5-15 °C for three weeks leading to the development of freezing tolerance, which is not seen in the C_4 type. This suggests that the C_3 variant of this species may demonstrate superior performance compared to the C_4 one, primarily due to its ability to withstand freezing damage rather than achieving higher photosynthetic rates. However, although the acclimation enables

plants to survive, it does not protect their photosynthetic system. While there have been physiological studies conducted on *A. semialata*, these were limited to only two genotypes representing the C_3 and C_4 types, and there is a notable absence of research on photoprotection among a diversity of different genotypes.

To broaden our understanding of how photodamage in *Alloteropsis* populations relates to their photosynthetic pathway and geographic provenance under chilling with high-light conditions, we treated 12 genotypes of *A. semialata*, each with distinct geographical histories, with the combination of environmental stressors: high-light intensity and a two-phase chilling treatment, over 14 days. We then measured and compared three photosynthetic parameters — PSII efficiency (Phi2), energy going through NO processes (PhiNO), and energy dissipated via NPQ (PhiNPQ) — as well as leaf greenness, indicated by the SPAD index. We hypothesised that C_4 photosynthesis is not inherently more sensitive to chilling than non-C_4 types. Instead, we hypothesised that evolutionary history would be more important, such that environmental tolerance is more closely related to geographic history than to photosynthetic pathways. We therefore expected that C_3, C_3-C_4, and C_4 plants would respond similarly to low temperatures, but plants from cooler climates would be more tolerant than those from warmer ones.

3. Materials and methods

Plant materials and growth conditions.

In this study, 12 genotypes of *Alloteropsis semialata* from different geographical origins were used (Table 5.1). All plants were clonally propagated from mother plants that were grown in a greenhouse at the Arthur Willis Environment Centre (AWEC) (University of Sheffield, Sheffield, UK). The growth conditions were set at 60% RH, 25/20 °C day/night temperatures, and 500 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) over a 14 h photoperiod at ambient CO_2 concentrations. Each propagated plant was cultivated in a separate container and kept in the greenhouse under these growth conditions for 10 weeks.

After clonal propagation, all plants were moved to grow in a controlled-environmental Conviron PGR15 plant growth chamber (Conviron Ltd, Winnipeg, Canada) to acclimate them to the environment of growth chamber. Environmental conditions were set with a 12-hour photoperiod (Light-on and -off at 5.00 a.m. and 5.00 p.m., respectively) with a photon flux density (PFD) of 500 μmol m^{-2} s^{-1}, measured at the height of the plants. The relative humidity was maintained at 50/60% day/night with the day/night temperature of 25/20 °C (Table S5.1). The plants were acclimated to these conditions for 10 days.

Before starting the chilling treatment, plants from both control and chilling treatments were acclimated to a high-light condition in the controlled-environment growth chambers with a photon flux density (PFD) of 800 μmol m^{-2} s^{-1} and a day/night temperature of 25/20 °C for 14 days. Then, the control treatment was treated with a day/night temperature of 25/20 °C for another 14 days, while the chilling treatment was treated with a day/night temperature of 15/10 °C for 7 days followed by a dropped day/night temperature of 10/5 °C for another 7 days. The measurements were done on different days after chilling (DAC) every two days after lights-on for 5 hours. Finally, leaf samples were collected at 15 days after chilling for future study (Figure 5.1).

Table 5.1 The 12 genotypes of *Alloteropsis semialata* that were used in this study.

Genotypes	Photo. type	Geographical Information				Bio Rep.	
		Country	Latitude	Longitude	MAT (°C)	Ctrl.	Chil.
KWT3-3	C_3	South Africa	-32.70	27.53	27.53	3	3
MDG1-2	C_4	South Africa	-25.76	29.47	16.77	2	3
TWN10-2	C_4	Taiwan	24.47	120.72	20.88	1	1
ZAM15-03-03	C_3- C_4	Zambia	-10.23	29.83	20.26	2	2
ZAM17-27	C_4	Zambia	-17.03	26.70	19.65	3	3
ZIM15-03-f2	C_3	Zimbabwe	-18.78	32.74	14.97	3	3
CAM-16-01-02	C_4	Cameroon	5.93	10.62	22.72	2	2
L01A	C_3- C_4	Tanzania	-5.63	32.69	23.50	1	1
MAJ3-1	C_4	Madagascar	-15.67	46.37	26.61	3	3
TAN16-02-03C	C_4	Tanzania	-9.04	32.48	21.81	3	3
ZAM15-02-01	C_4	Zambia	-10.18	30.95	20.78	3	3
BF-2	C_4	Burkina Faso	10.85	-4.83	26.58	1	1

Note: Abbv.: Photo. Type = photosynthetic type, Bio Rep. = number of biological replications at the beginning of the experiment, Ctrl. and Chil. Mean control and chilling treatments, respectively.

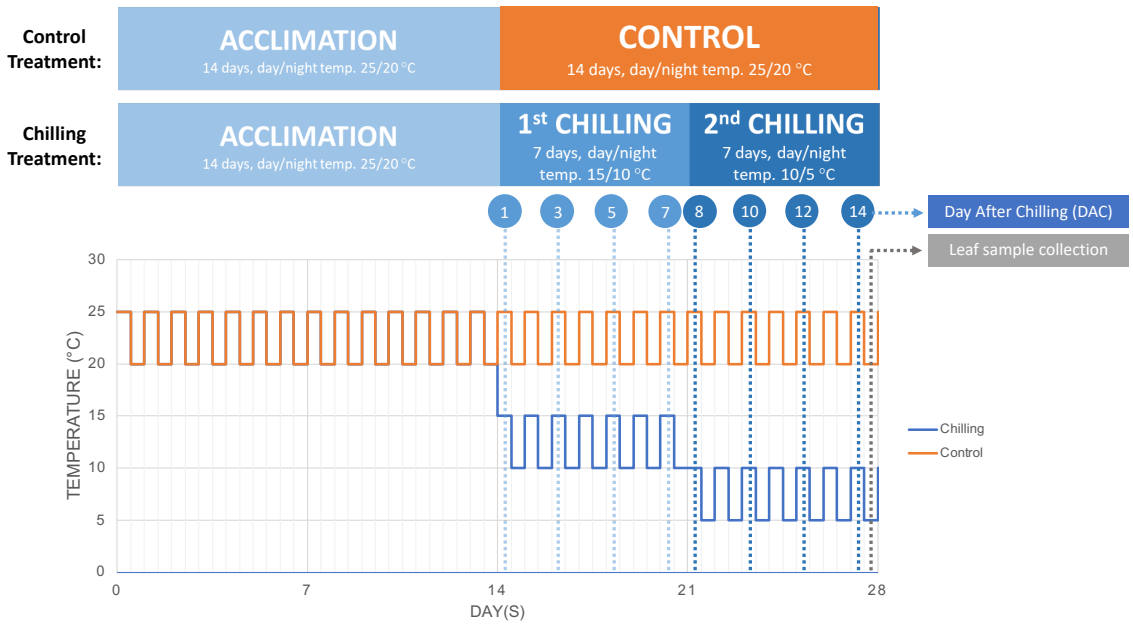

Figure 5.1 Experimental design in this study applying a short-term chilling treatment in combination with high-light intensity. Note: the high light treatment was applied for the whole duration of this experiment.

Measurements of photosynthetic, environmental and leaf parameters

The hand-held MultispeQ V2.0 (PhotosynQ, USA) was used to measure photosynthetic, environmental and leaf parameters (Table S5.2). The photosynthetic parameters measured included the quantum yield of photosystem II (Phi2 or Φ_{II}), the ratio of incoming light that is lost via non-regulated processes (PhiNO or Φ_{NO}), and the ratio of incoming light that goes toward non-photochemical quenching (PhiNPQ or Φ_{NPQ}). Photobleaching was measured via the relative chlorophyll content, which was measured as the index SPAD, and leaf temperatures were measured in a unit of degrees Celsius (°C). Associated measured environmental parameters comprised light intensity (PAR) and ambient temperature (°C), relative humidity (%), and pressure (mbar).

The measurements were done on 1-3 plants per genotype depending on plant stock availability. Three leaves of each plant, the second to fourth leaves from the top, were measured with three technical replicates. The days of data collection are defined as days after chilling (DAC), which are shown in Figure 5.1. Briefly, the first measurements were done on the first day after starting the first chilling treatment (DAC

1) after light-on for 5 hours and then repeated every two days for seven days (DAC 3, 5, and 7). For the second chilling treatment, the measurements were also repeated every two days at DAC 8, 10, 12, and 14.

To compare the effect of chilling treatment on the photosynthetic efficiency of each genotype, we calculated a log response ratio between chilling and control treatments. The formula for each calculation is shown below.

$$\Phi_{II} = log\left(\frac{\Phi_{II_{chilling}}}{\Phi_{II_{control}}}\right) \quad \text{Equation (5.1)}$$

$$\Phi_{NO} = log\left(\frac{\Phi_{NO_{chilling}}}{\Phi_{NO_{control}}}\right) \quad \text{Equation (5.2)}$$

$$\Phi_{NPQ} = log\left(\frac{\Phi_{NPQ_{chilling}}}{\Phi_{NPQ_{control}}}\right) \quad \text{Equation (5.3)}$$

$$SPAD = log\left(\frac{SPAD_{chilling}}{SPAD_{control}}\right) \quad \text{Equation (5.4)}$$

Statistical analyses

Phylogenetic generalised linear models (PGLS) with an interaction term were fitted by using the function pgls( ) in R version 4.2.2. This analysis aimed to investigate the combined impact of different types of photosynthetic pathways and geographical provenance, represented by MAT, on various photosynthetic parameters (Phi2, PhiNO, and PhiNPQ), and SPAD on four different times points; days after chilling (DAC) 1, 7, 8, and 14, which was done on the mean for each genotype. Photosynthetic types were classified into two groups, which are C_4 and non-C_4, including C_3 and C_3-C_4 intermediate. The dependent variables are the log response ratios of each particular photosynthetic parameter and SPAD, while the independent variables consist of photosynthetic types and MAT. The PGLS formulas used were shown as follows:

$$pgls(PhotosynQ\_parameter \sim (photosynthetic\_type * MAT)$$

4. Results

All genotypes of *A. semialata* exhibited decreased photosynthetic efficiency of PSII following exposure to chilling temperatures and high-light conditions.

Overall, Phi2 differed in the chilling treatment compared with the control across all genotypes (Figure 5.2 (A) and Supplement: Figure S5.1 (A)). During the initial chilling period at 15/10 °C for 7 days, most genotypes showed a continuous decline in Phi2. However, five genotypes, including one C_4 genotype (BF2) and four non-C_4 genotypes (KWT3-3, ZIM15-03-f2, MDG1-2, and LO1A), exhibited a slight increase in Phi2 on DAC 7, albeit still lower than at the first day of the experiment (DAC 1). This suggests that these genotypes can partially recover the efficiency of photosystem II (PSII) under chilling conditions. Subsequently, upon commencing the second phase of the experiment at DAC 8, Phi2 of all genotypes sharply declined and continued to decrease until the end of the experiment at DAC 14 (Figure 5.2 (A)). These findings indicate that while genotypes initially respond differently to chilling, prolonged exposure to lower temperatures damages all genotypes to a similar extent over time.

We further examined the relationship and interaction effect of photosynthetic pathways (C_4 and non-C_4) and mean annual temperature (MAT) on Phi2 at four different time points during the experiment. Our phylogenetic generalised least squares (PGLS) analysis revealed a negative correlation between C_4 photosynthesis and the log response ratio of Phi2 at DAC14 with a strong statistically significant p-value of 0.014 (Figure 5.3 (D) and Supplement: Table S5.3). However, there is no statistically significant relationship between individual photosynthetic pathways or MAT and Phi2 at the other time points, nor did it detect an interaction effect between both predictors, photosynthetic pathway, and MAT on Phi2 (Figure 5.3 (A-D) and Supplement: Table S5.3). These results indicate that changes in Phi2 due to chilling with high-light conditions are not influenced by the geographic provenance or photosynthetic pathway of specific *Alloteropsis* genotypes, except for C_4 photosynthesis on the last day of the experiment, which negatively influenced Phi2.

Similar to Phi2, all *A. semialata* genotypes exhibited reduced energy release through non-regulated processes (PhiNO) following chilling treatment.

PhiNO levels decreased across all *Alloteropsis* genotypes to varying degrees as temperatures began to decline (Figure 5.2 (B) and Supplement: Figure S5.1 (B)). Between days 5 and 7, two C_4 genotypes, CAM16-01-02 and MDG1-2, demonstrated a continuous decline in PhiNO, while others were able to recover partially. However, PhiNO levels remained low compared to the first day of the experiment. This suggests that in the majority of the *Alloteropsis* genotypes energy expenditure in non-regulated processes increases again by day 7, whereas CAM16-01-02 and MDG1-2 cannot. However, upon the beginning of the second phase of chilling treatment on day 8, PhiNO continued to decline steadily until the experiment ended on day 14, with all genotypes exhibiting similar PhiNO levels (Figure 5.2 (B)). These results suggest that although genotypes exhibit varied initial responses to chilling, extended exposure to lower temperatures eventually leads to comparable damage across all genotypes.

Additionally, we investigated whether the photosynthetic pathways and/or geographical provenance influence the variation in PhiNO under chilling with high-light conditions. Interestingly, we observed a significant negative correlation between the C_4 photosynthetic pathway and PhiNO on DAC 14, with a strong statistical significance (p-value = 0.007) (Figure 5.3 (H) and Supplement: Table S5.4). This implies that the genotypes of C_4 photosynthesis tend to spend energy less on non-regulatory processes, which means that they are more vulnerable to prolonged chilling with high-light conditions. However, there is no statistical relationship between the variation in PhiNO and either individual non-C_4 photosynthetic types or MAT. Furthermore, we found no combined effect of photosynthetic types and MAT on the change in PhiNO.

The NPQ mechanism is universally employed across all genotypes of *A. semialata* in response to chilling under high-light conditions.

To assess the utilisation of the NPQ mechanism in response to changing temperature conditions, PhiNPQ was calculated using the PhotosynQ formula provided in Table S5.2. Overall, our results indicate positive log response ratios of PhiNPQ across all genotypes, except for KWT3-3 on day 1, with variations observed among genotypes (Figure 5.2 (C) and Supplement: Figure S5.1 (C)). This signifies higher PhiNPQ values in chilled plants compared to the control group, indicating the utilisation of the NPQ mechanism across all genotypes with varying degrees of response to chilling with high-light conditions.

During the initial chilling treatment regardless of statistical significance, the non-C_4 genotypes, except KWT3-3, exhibited higher PhiNPQ levels than the C_4 genotypes (Figure 5.2(C)). PhiNPQ remained relatively stable throughout the first chilling treatment, with only CAM16-01-02, a C_4 genotype, displaying an increase over time. Following the commencement of the second phase, PhiNPQ alterations continued to rise in most genotypes until day 14, except for ZAM15-03-03, a non-C_4 genotype, which exhibited a decline in PhiNPQ over time (Figure 5.2(C)). This suggests that most *Alloteropsis* genotypes utilise NPQ mechanisms in response to chilling with high-light conditions with varying degrees of response. However, prolonged chilling with temperature decrease may disrupt the NPQ mechanism, potentially leading to plant damage.

Furthermore, we examined the relationship between photosynthetic pathways, MAT, and changes in PhiNPQ. Our PGLS analysis revealed a positive correlation between the C_4 photosynthetic type and PhiNPQ alterations on the final day of the experiment (DAC 14), with a marginally significant p-value of 0.079 (Figure 5.3 (L) and Supplement: Table S5.5). This suggests that the NPQ mechanisms are important for the genotypes with C_4 photosynthesis when they experience prolonged chilling with high-light conditions and implies that the C_4 genotypes use different mechanisms of regulation under chilling temperature. However, neither the C_3 photosynthetic type nor MAT demonstrated statistical significance in relation to PhiNPQ changes under chilling

with high-light conditions, nor did the interactive effects of both photosynthetic type and MAT (Supplement: Table S5.5).

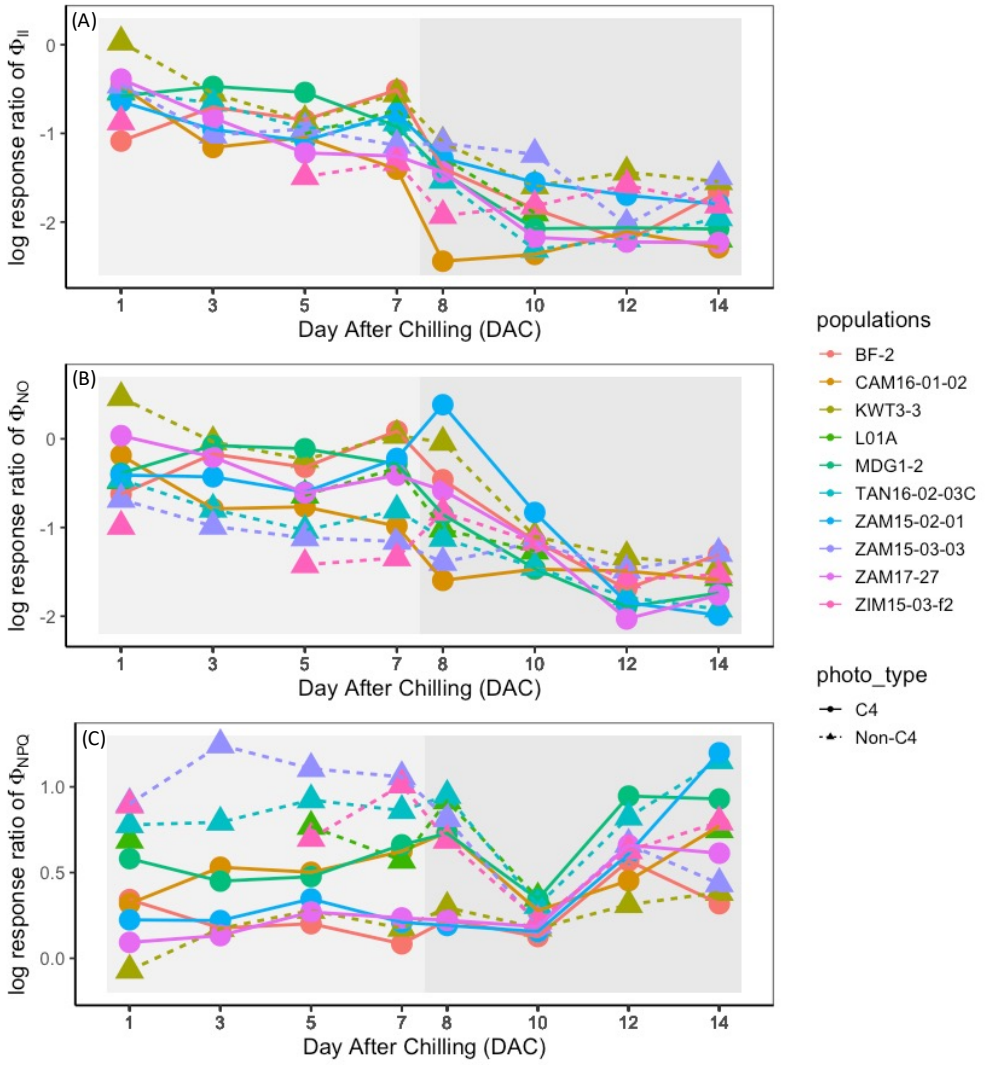

Figure 5.2 The responses of 12 genotypes of *A. semialata* in each particular photosynthetic parameter, represented by the log response ratio of Phi2 (A), PhiNO (B), and PhiNPQ (C), during 14 days of two phases of chilling with high-light treatments. The background colour distinguishes between the first and second phases of the chilling treatment, indicated by light and dark grey, respectively.

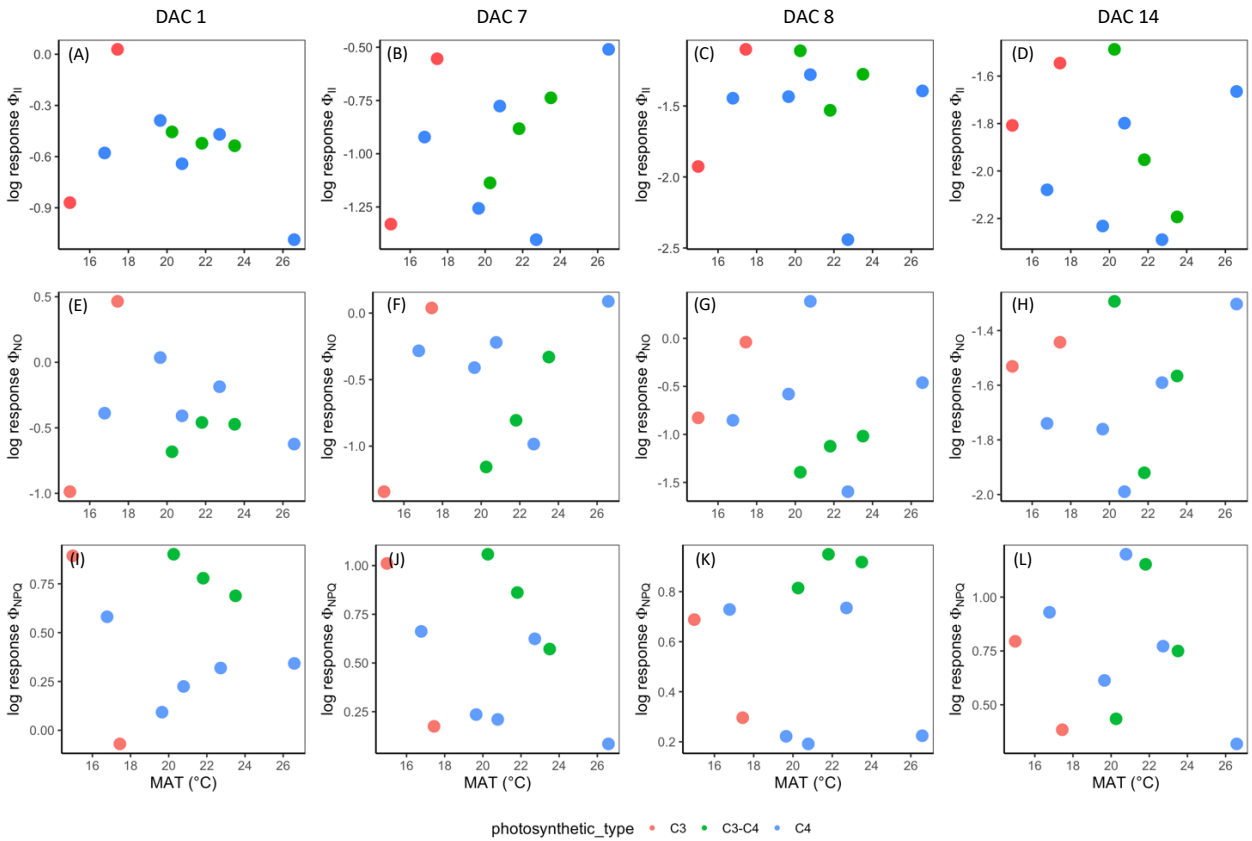

Figure 5.3 Responses of 12 genotypes of *A. semialata* to each specific photosynthetic parameter at different time points after exposure to chilling with high-light conditions for 14 days. Photosynthetic parameters include Phi2 (A-D), PhiNO (E-H), and PhiNPQ (I-L). The initial chilling phase with a day/night temperature of 15/10 °C spans from Day After Chilling (DAC) 1 (A, E, I) to DAC 7 (B, F, J), while the second phase of chilling treatment with a day/night temperature of 10/5 °C extends from DAC 8 (C, G, K) to DAC 14 (D, H, L). Abbreviations: DAC, Day After Chilling; MAT, Mean Annual Temperature.

Low temperatures induce colour changes in the leaves of *A. semialata* with varying responses observed among different genotypes

To evaluate chlorophyll content, we measured the SPAD index, a relative measure nonlinearly linked to chlorophyll concentration (Uddling et al., 2007). Higher SPAD values indicate greater chlorophyll concentration and greener leaves. We hypothesised that if plant greenness is adversely affected by high-light conditions or sensitive to them, SPAD indexes of control plants would decrease over time, while unaffected plants would maintain constant SPAD values. Our results revealed a decrease in SPAD over time during both chilling phases in five genotypes, including four C_4 genotypes (CAM16-01-02, ZAM17-27, MDG1-2, and BF-2) and one non-C_4 genotype (LO1A) (Figure 5.4 and Supplement: Figure S5.2). This suggests a reduction in leaf greenness compared to the experiment's onset. Conversely, two non-C_4 genotypes (ZAM15-03-03 and TAN16-02-03c) displayed nearly constant SPAD values over time (Figure 5.4 and Supplement: Figure S5.2), indicating minimal impact from chilling and high-light intensity. Interestingly, one C_4 genotype (ZAM15-02-01) and two non-C_4 genotypes (KWT3-3 and ZIM15-03-f2) exhibited increased SPAD values over time, suggesting potential mechanisms to maintain chlorophyll content or promote new leaf growth under chilling with high-light conditions.

We further explored the relationship between photosynthetic pathways, MAT, and SPAD changes during exposure to chilling with high-light conditions. Notably, PGLS analysis revealed a negative correlation between the non-C_4 photosynthetic type and SPAD change on the first day of the second chilling treatment (DAC 8), with a marginally significant p-value of 0.099 (Supplement: Table S5.6). However, statistical significance was not observed for the relationship between photosynthetic pathways, MAT, and SPAD alteration at other time points, nor for the combined effect of photosynthetic type and MAT on SPAD change.

Furthermore, we observed noticeable changes in leaf physical characteristics throughout the chilling treatment. For instance, MDG1-2 (Supplement: Figure S5.3 (A-C)) exhibited leaf tip necrosis and yellowing leaves, likely due to burning at the tips. Additionally, leaves of ZAM15-03-03 (Supplement: Figure S5.3 (D-F)) displayed red colouration at the tips, attributed to anthocyanin accumulation.

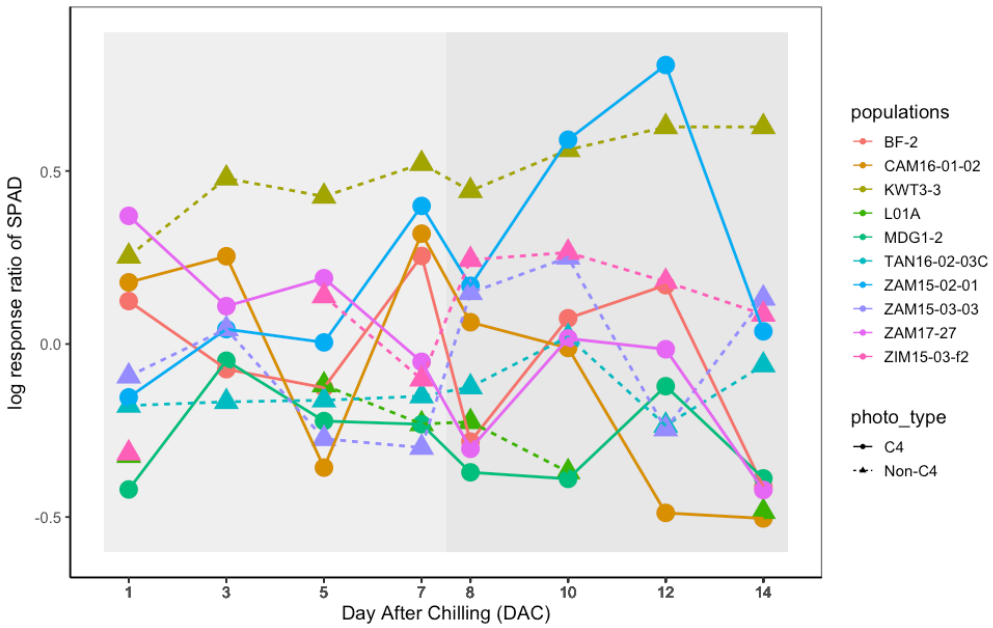

Figure 5.4 The responses of 12 genotypes of *A. semialata* leaf greenness, represented by the log response ratio of SPAD, during 14 days of two phases of chilling with high-light treatments.

5. Discussion

In this study, we have explored how different genotypes of *Alloteropsis* respond to low temperatures under high-light conditions. Overall, regardless of photosynthetic pathways, geographical history, and statistical tests, all *Alloteropsis* genotypes exhibited a response to low temperatures by decreasing the efficiency of photosystem II (Phi2) and the energy expended via non-regulated processes (PhiNO), whereas regulated energy dissipation via NPQ mechanisms (NPQ) increased with different levels of response among genotypes. These indicate that these responses are common among *Alloteropsis* genotypes and NPQ may be one of the key protective mechanisms preventing C_4 plants in this species from photoinhibition under low temperatures with high-light conditions. After starting the second phase of chilling treatment with the drop in temperature to a day/night temperature of 10/5 °C, Phi2 and PhiNO in all genotypes obviously dropped with different levels and continued dropping until the end of the experiment. These results imply that *A. semialata* may have a slow growth at temperatures below 10 °C and may start to damage as shown in physical and colour changes in leaves. This phenomenon can be explained by the mean annual temperature (MAT) of all 12 genotypes in this study which ranges between 15 and 25 °C (Table 5.1) and is comparatively warmer than 10 °C. Thus, the plants possibly stop or slow down growth under suboptimal temperatures for each particular genotype. Notably, Osborne et al. (2008) reported similar findings, noting that both C_3 and C_4 genotypes exhibited declining photosynthetic rates and growth retardation at temperatures ≤17 °C, likely due to susceptibility to photodamage.

We further asked whether the intraspecific tolerance to low temperatures with high-light conditions among *Alloteropsis* genotypes is influenced by their photosynthetic pathways, geographical origins, or neither. Previous research indicates that although C_4 plants originated from tropical and subtropical regions (Hattersley, 1983; Ehleringer et al., 1987; Cabido et al., 2008), they have demonstrated an ability to adapt and thrive under low temperatures (Watcharamongkol et al., 2018). Furthermore, a study involving grasses from various lineages suggested that freezing tolerance is more closely associated with life history and ecological preferences rather than photosynthetic pathways (Liu et al., 2013). These findings indicate that the

susceptibility of C_4 plants to chilling damage may arise from their tropical/subtropical origin rather than an inherent vulnerability of the C_4 pathway to chilling. Consequently, we hypothesised that geographical provenances may have more impact on these environmental tolerances over the photosynthetic pathways. Unexpectedly, we did not observe any significant influence of geographical history on photosynthesis under chilling conditions with high light in our study. Instead, our data suggest there are consistent physiological differences in the response of C_4 plants to chilling compared to non-C_4 plants.

Interestingly, our study reveals the influence of the C_4 photosynthetic pathway on the partitioning of photosynthetic energy usage on photochemistry after prolonged growing under chilling with high-light conditions. This indicates that C_4 is more vulnerable to low temperatures (at a day/night temperature of 10/5 °C) with high-light conditions than non-C_4 genotypes, including both C_3 and C_3-C_4 pathways. This observation is consistent with previous research that both C_3 and C_4 varieties of *A. semialata* exhibited distinct responses when exposed to prolonged chilling, including decreased net leaf photosynthesis when temperature dropped below 10 °C. Specifically, C_3 leaves developed freezing protective mechanisms through cold acclimation, while such mechanisms were notably absent in the C_4 counterparts (Osborne et al., 2008).

Our findings indicate a significant correlation between the C_4 photosynthetic pathway and alterations in PhiNPQ on the final day of the experiment (DAC 14), highlighting the importance of NPQ mechanisms in the adaptation and survival of C_4 genotypes under prolonged chilling with high-light conditions. Interestingly, this correlation was not observed for the non-C_4 photosynthesis, suggesting potential differences in regulatory mechanisms between the two photosynthetic pathways. This observation aligns with a previous study examining the impacts of drought on *A. semialata*, which compared C_3 and C_4 genotypes. Despite both types of plants exhibiting a decrease in Phi2, indicating a similar response to excess energy, the study emphasised the critical role of mechanisms dissipating excess energy as heat and maintaining photosynthetic balance specifically in C_4 plants (Ripley et al., 2007). Therefore, these findings underscore the importance of NPQ mechanisms in the unique adaptation strategies employed by C_4 plants to cope with environmental stressors such as chilling and drought.

Moreover, we have found that neither photosynthetic pathways nor geographical origins influenced leaf greenness. However, there is a positive and marginally significant relationship between the non-C_4 pathways and the change in the SPAD index on day 8, the first day of the second phase of chilling treatment. This indicates that on day 8 the leaf chlorophyll content was increased, suggesting that the non-C_4 genotypes may be able to generate new leaves, or they may have some pathways to increase their leaf chlorophyll content which may be involved in maintaining or compensating their photosynthetic efficiency under this suboptimal growth conditions. However, this finding is contradicted by the response to low temperatures observed previously in various grasses, which often show a suppression in chlorophyll biosynthesis under chilling conditions. For instance, in C_3 rice seedlings, δ-aminolevulinic acid (ALA) synthesis, which is the early step of chlorophyll was inhibited by low temperatures (Zhao et al., 2020).

Furthermore, we observed distinct physical alterations in leaves, including necrosis, chlorosis, and red colouration, particularly evident in certain genotypes, notably the C_4 and C_3-C_4 types. Notably, in the C_4 genotype from South Africa MDG1-2, leaf necrosis and chlorosis occurred after exposure to chilling conditions with high-light intensity for 14 days. These leaf alterations are common responses in plants sensitive to such suboptimal growth conditions, indicating photodamage that may lead to PSII photoinhibition (Szymańska et al., 2017). Similar damage patterns have been observed in various grass species. For instance, buffelgrass (*Cenchrus ciliaris*), a C_4 tropical grass, exhibited signs of foliar damage including wilt, chlorosis, and necrosis when exposed to -5 °C temperatures, followed by burnt tips and twisting after thawing in sunlight (Parera et al., 2019). Moreover, these alterations are consistent with our results that genotypes with C_4 were more vulnerable than the others, as shown in the decreased Phi2 and PhiNO and increased PhiNPQ on day 14 after chilling.

We observed red coloration in ZAM15-03-03, a Zambian C_3-C_4 genotype, which was developed from day 7 to day 14. This red colour is typically caused by the accumulation of anthocyanin in the leaves enhancing the antioxidant capacities to reduce oxidative stress induced by low temperatures or high-intensity light (Chalker-Scott, 1999; Sivankalyani et al., 2016). Similar responses have been reported in various grass species, such as *Zoysia japonica* thriving under low-temperature conditions (Jin

et al., 2022) and rough bluegrass (*Poa trivialis L.*) responding to high-intensity light (Petrella et al., 2016).

The observed physical alterations suggest specificity to particular species and appear unrelated to either photosynthetic pathways or geographical origins. However, due to limitations in biological replication for some genotypes, further studies are needed to elucidate these findings. Furthermore, as our observations lack comparisons between genotypes, measuring pigment content is essential to understand the causes of colour changes.

Several limitations must be addressed in this study. Firstly, the narrowness of grass leaves could potentially affect the accuracy of measurements as the leaves could not cover the sensor area for the measurement of the equipment. This could potentially introduce inaccuracies in photosynthetic parameter measurements. Secondly, the measurement of NPQ was not a direct measurement with a dark adaptation, but PhiNPQ was obtained from a calculation, which is proportional to Phi2 and PhiNO (a formula shown in Supplement: Table S5.2). This suggests that the energy that is dissipated via other regulatory mechanisms, excluding NO, and NPQ, may be ignored from our study. Thirdly, although this experiment was done by one person with one measuring device to reduce human and machine errors, this may have led to a longer duration in the time of the measurement. The variations in the time of measurement could have impacted the measured parameters, particularly NPQ, which change during the time of day. Additionally, we have found that after using the equipment over a prolonged time, the measured ambient and leaf temperatures increased by about 3-4 ºC. This might be influenced by the increase in equipment temperature; however, we did not measure the equipment temperature directly to confirm. These limitations underscore the need for caution in interpreting the results and suggest directions for future research to address these confounding factors.

6. Conclusions

In this study, we aimed to investigate the intraspecific physiological response of *A. semialata* to low temperatures under high-light conditions, focusing on photodamage to photosystem II (PSII) and photoprotection mechanisms through non-regulated processes (NO) and non-photochemical quenching (NPQ). We also examined whether environmental tolerance in this species is influenced by photosynthetic pathways and geographical history.

Our findings reveal that chilling with high-light conditions led to decreased PSII efficiency and reduced energy dissipation on NO processes, while NPQ, a photoprotective mechanism, increased across all genotypes with varying levels of response. These effects deteriorated under more intense and prolonged chilling conditions, indicating suboptimal growth conditions for this species.

Surprisingly, we did not observe a significant influence of geographical provenance on tolerance to these environmental conditions. However, we unexpectedly found a relationship between C_4 photosynthetic pathways and alterations in all three photosynthetic parameters (Phi2, PhiNO, and PhiNPQ), suggesting that C_4 genotypes are particularly vulnerable to prolonged chilling under high-light conditions.

Furthermore, we found no effects of geographical history or photosynthetic pathways on leaf greenness, as represented by the SPAD index. However, PGLS analysis revealed a marginally significant positive relationship between SPAD and non-C_4 photosynthetic pathways on the first day of the second chilling phase (DAC 8), suggesting that non-C_4 genotypes, including both C_3 and C_3-C_4 pathways, may have mechanisms to increase leaf chlorophyll content or promote new leaf growth under these suboptimal conditions.

Finally, we observed physical changes in leaves, such as necrosis and the accumulation of anthocyanin, particularly in some C_4 genotypes. These findings provide valuable insights for further study on how C_4 genotypes respond to chilling under high-light conditions.

7. Acknowledgements

I would like to all members of Prof. Colin Osborne and Dr. Luke Dunning Lab for teaching me how to propagate plants and helping me water plants. This work was funded by a studentship awarded by the Royal Thai Government.

8. Supplementary data

Table S5.1 Growth conditions for plants used in this study.

Status	Growth Conditions	Treatment groups	
		Control	Chilling
Pre-experiment (10 days)	D/N temp.	25/20 °C	
	Light intensity	500 µmol m^{-2} s^{-2}	
	D/N RH	50/60%	
	Light cycle	12-hr photoperiod	
High-light acclimation (14 days)	D/N temp.	25/20 °C	
	Light intensity	800 µmol m^{-2} s^{-2}	
	D/N RH	50/60%	
	Light cycle	12-hr photoperiod	
1st Chilling treatment (7 days)	D/N temp.	25/20 °C, 7days	15/10 °C, 7 days
	Light intensity	800 µmol m^{-2} s^{-2}	
	D/N RH	50/60%	
	Light cycle	12-hr photoperiod	
2nd Chilling treatment (7 days)	D/N temp.	25/20 °C, 7 days	10/5 °C, 7 days
	Light intensity	800 µmol m^{-2} s^{-2}	
	D/N RH	50/60%	
	Light cycle	12-hr photoperiod	

Abbv: D/N temp. = day/night temperatures, D/N RH = day/night relative humidity

Table S5.2 PhotosynQ parameters measured in this study.

Parameters	Calculation	Note
Yield and electron transfer		
Phi2 (Φ_{II}) (Quantum Yield)	$\Phi_{II}= \frac{F'_m - F_s}{F'_m}$	The portion of light energy absorbed by PS II, utilised for photochemistry to produce ATP, NADPH, and ultimately sugar, ranges from 0 to 0.82; F'_m = the maximum fluorescence yield during a saturating light pulse applied to light-exposed leaves, and F_s = the fluorescence emission in light-adapted samples.
PhiNO (Φ_{NO}) (Non-regulatory energy dissipation)	$\Phi_{NO} = \frac{F_s}{F_m}$	The fraction of incoming light lost due to unregulated processes; F_m = the maximum fluorescence yield during a saturating light pulse applied to dark-adapted leaves.
Non photochemical quenching (NPQ)		
PhiNPQ (Φ_{NPQ})	$\Phi_{NPQ} = 1 - \Phi_{II} - \Phi_{NO}$	The proportion of incoming light directed towards non-photochemical quenching.
Environmental Parameters		
Light Intensity (PAR)	-	Photosynthetically active radiation.; µmol photon * m-2 * s-1
Ambient Temperature	-	Ambient temperature in degree Celsius
Ambient Humidity	-	Relative humidity in percent (%)
Ambient Pressure	-	Atmospheric pressure (mbar) ** This value is not corrected to sea level as found in weather reports.
Leaf Parameters		
Leaf Temperature	-	Temperature of leaf surface
Leaf Temperature Differential	Leaf Temp. – Ambient Temp.	** -ve number = the leaf is cooler than the surrounding air
SPAD	-	Special Products Analysis Division. A measure of leaf greenness

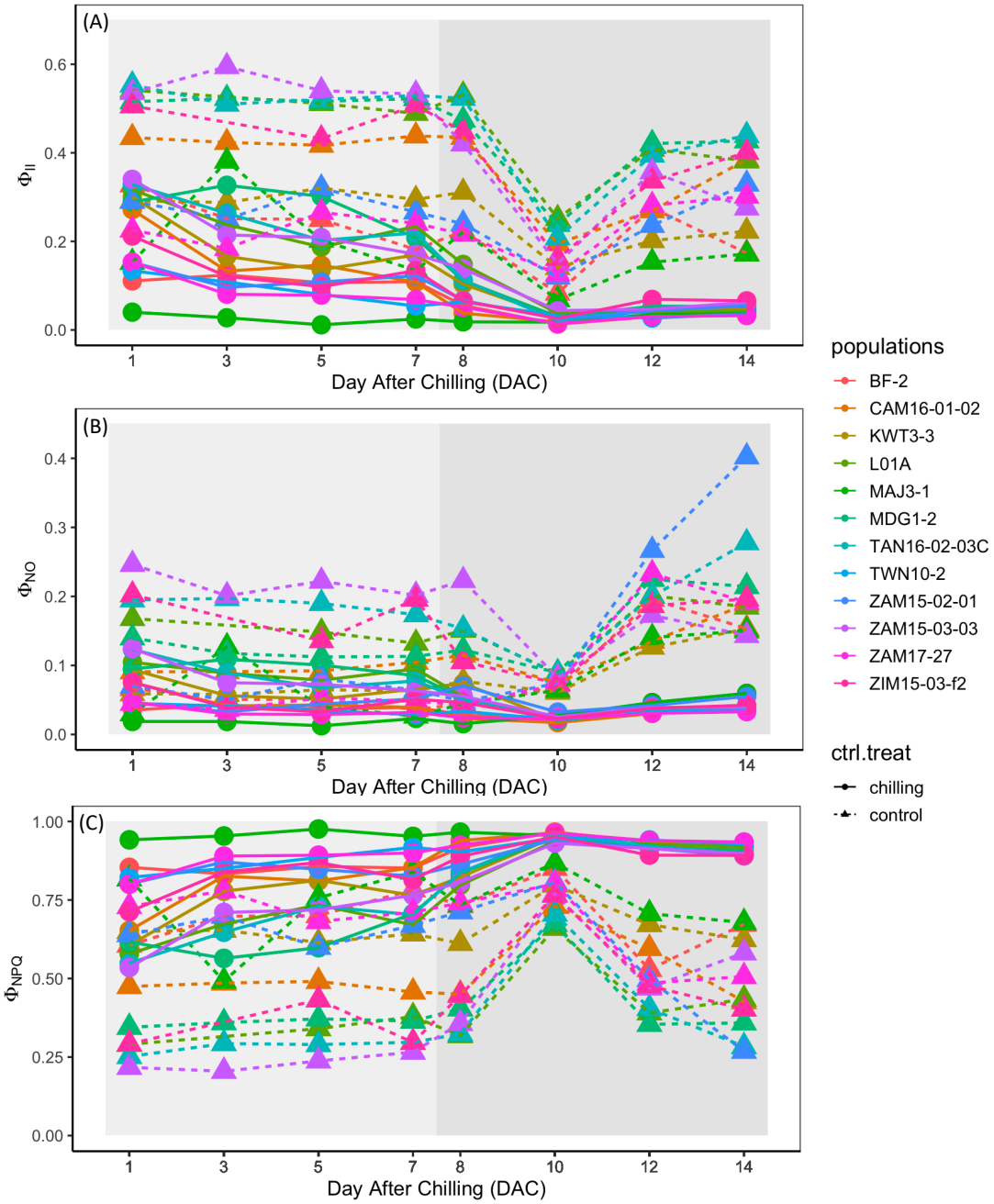

Figure S5.1 The responses of 12 genotypes of *A. semialata* in each particular photosynthetic parameter on Phi2 (A), PhiNO (B), and PhiNPQ (C), during 14 days of two phases of chilling with high-light treatments. The background colour distinguishes between the first and second phases of the chilling treatment, indicated by light and dark grey, respectively.

Table S5.3 Results of PGLS testing for the combination effects of photosynthetic types, including C_4 and non-C_4 pathways, and geographic provenance based on mean annual temperature (MAT) on the log response ratio of Phi2 at different time points after chilling treatment for 14 days.

DAC	Predictors	λ	Adjusted R^2	p-value	Estimate	t value	Pr(>|t|)
1	C_4	0.000	-0.081	0.549	0.488	0.541	0.608
	Non-C_4	0.000	-0.081	0.549	-1.131	-0.894	0.406
	MAT	0.000	-0.081	0.549	-0.053	0.255	0.255
	Non-C_4: MAT	0.000	-0.081	0.549	0.061	1.002	0.355
7	C_4	0.000	-0.263	0.774	-1.757	-1.668	0.146
	Non-C_4	0.000	-0.263	0.774	0.089	0.060	0.954
	MAT	0.000	-0.263	0.774	0.037	0.480	0.480
	Non-C_4: MAT	0.000	-0.263	0.774	0.001	0.014	0.990
8	C_4	0.000	-0.259	0.769	-1.101	-0.817	0.445
	Non-C_4	0.000	-0.259	0.769	-1.202	-0.635	0.549
	MAT	0.000	-0.259	0.769	-0.023	-0.373	0.722
	Non-C_4: MAT	0.000	-0.259	0.769	0.070	0.092	0.474
14	C_4	0.000	0.305	0.296	-2.809	-3.439	0.014 *
	Non-C_4	0.000	0.305	0.296	1.928	1.682	0.144
	MAT	0.000	0.305	0.296	0.037	0.362	0.362
	Non-C_4: MAT	0.000	0.305	0.296	-0.084	-1.526	0.180

Note: λ refers to Pagel's estimator of phylogenetics. Significance level: · , $p < 0.1$; *, $p < 0.05$; **, $p < 0.01$. Abbreviation: DAC; Day after chilling.

Table S5.4 Results of PGLS testing for the combination effects of photosynthetic types, including C_4 and non-C_4 pathways, and geographic provenance based on mean annual temperature (MAT) on the log response ratio of PhiNO at different time points after chilling treatment for 14 days.

DAC	Predictors	λ	Adjusted R^2	p-value	Estimate	t value	Pr(>|t|)
1	C_4	0.000	-0.414	0.944	0.342	0.242	0.817
	Non-C_4	0.000	-0.414	0.944	-0.873	-0.441	0.675
	MAT	0.000	-0.414	0.944	-0.031	-0.471	0.655
	Non-C_4: MAT	0.000	-0.414	0.944	0.036	0.376	0.720
7	C_4	0.000	-0.197	0.692	-0.817	-0.507	0.630
	Non-C_4	0.000	-0.197	0.692	-0.861	-0.381	0.717
	MAT	0.000	-0.197	0.692	0.021	0.286	0.785
	Non-C_4: MAT	0.000	-0.197	0.692	0.028	0.252	0.810
8	C_4	0.000	-0.304	0.824	-0.589	-0.291	0.781
	Non-C_4	0.000	-0.304	0.824	1.168	0.412	0.696
	MAT	0.000	-0.304	0.824	-0.002	-0.016	0.988
	Non-C_4: MAT	0.000	-0.304	0.824	-0.073	-0.530	0.614
14	C_4	0.000	0.079	0.370	-2.747	-4.074	0.007 **
	Non-C_4	0.000	0.079	0.370	1. 629	1.721	0.136
	MAT	0.000	0.079	0.370	0.050	1.606	0.159
	Non-C_4: MAT	0.000	0.079	0.370	-0.072	-1.578	0.166

Note: λ refers to Pagel's estimator of phylogenetics. Significance level: · , $p < 0.1$; *, $p < 0.05$; **, $p < 0.01$. Abbreviation: DAC; Day after chilling.

Table S5.5 Results of PGLS testing for the combination effects of photosynthetic types, including C_4 and non-C_4 pathways, and geographic provenance based on mean annual temperature (MAT) on the log response ratio of PhiNPQ at different time points after chilling treatment for 14 days.

DAC	Predictors	λ	Adjusted R^2	p-value	Estimate	t value	Pr(>|t|)
1	C_4	0.000	-0.077	0.545	0.568	0.542	0.607
	Non-C_4	0.000	-0.077	0.545	-0.362	-0.246	0.814
	MAT	0.000	-0.077	0.545	-0.012	-0.247	0.813
	Non-C_4: MAT	0.000	-0.077	0.545	0.034	0.071	0.649
7	C_4	0.000	0.063	0.386	1.226	1.197	0.276
	Non-C_4	0.000	0.063	0.386	-0.401	-0.279	0.789
	MAT	0.000	0.063	0.386	-0.040	-0.852	0.427
	Non-C_4: MAT	0.000	0.063	0.386	0.036	0.517	0.624
8	C_4	0.000	0.230	0.232	1.034	1.300	0.241
	Non-C_4	0.000	0.230	0.232	-1.311	-1.175	0.285
	MAT	0.000	0.230	0.232	-0.029	-0.781	0.465
	Non-C_4: MAT	0.000	0.230	0.232	0.080	1.487	0.188
14	C_4	0.000	-0.104	0.576	1.990	2.111	0.079 .
	Non-C_4	0.000	-0.104	0.576	-1.187	-1.373	0.219
	MAT	0.000	-0.104	0.576	-0.057	-1.313	0.237
	Non-C_4: MAT	0.000	-0.104	0.576	0.084	1.318	0.236

Note: λ refers to Pagel's estimator of phylogenetics. Significance level: · , $p < 0.1$; *, $p < 0.05$; **, $p < 0.01$. Abbreviation: DAC; Day after chilling.

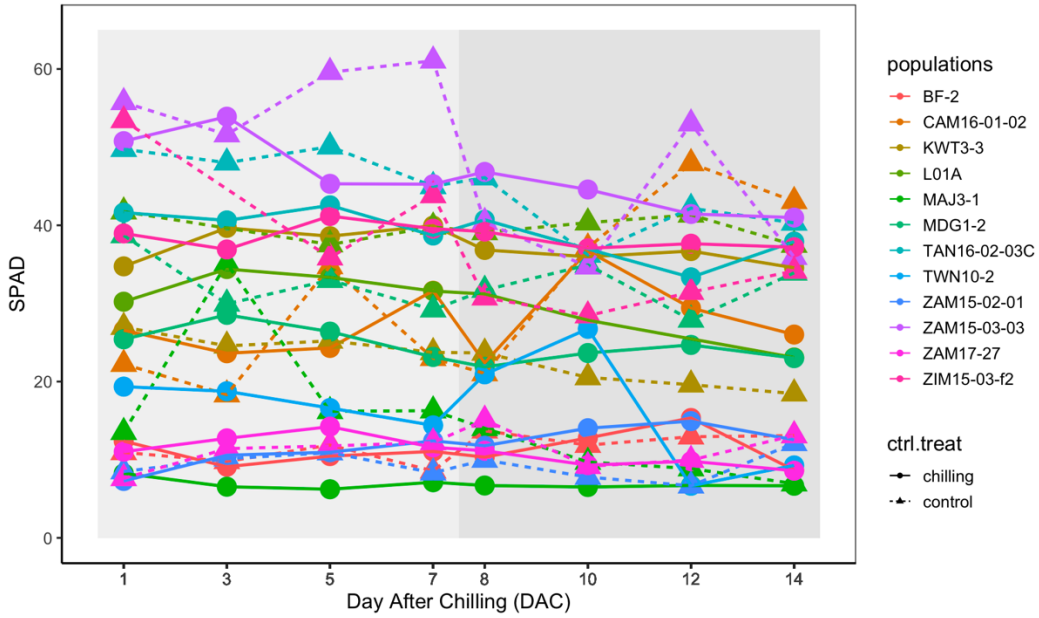

Figure S5.2 The SPAD index measurement of 12 genotypes of *A. semialata* during 14 days of two phases of chilling-high-light treatments.

Table S5.6 Results of PGLS testing for the combination effects of photosynthetic types, including C_4 and non-C_4 pathways, and geographic provenance based on mean annual temperature (MAT) on the log response ratio of SPAD at different time points after chilling treatment for 14 days.

DAC	Predictors	λ	Adjusted R^2	p-value	Estimate	t value	Pr(>|t|)
1	C_4	0.000	-0.119	0.595	-0.891	-1.053	0.333
	Non-C_4	0.000	-0.119	0.595	1.097	0.924	0.391
	MAT	0.000	-0.119	0.595	0.043	1.089	0.318
	Non-C_4: MAT	0.000	-0.119	0.595	-0.060	-1.043	0.337
7	C_4	0.000	0.097	0.352	-0.949	-1.128	0.303
	Non-C_4	0.000	0.097	0.352	1.773	1.501	0.184
	MAT	0.000	0.097	0.352	0.051	1.306	0.239
	Non-C_4: MAT	0.000	0.097	0.352	-0.096	-1.674	0.145
8	C_4	0.000	0.309	0.172	-0.421	-0.625	0.555
	Non-C_4	0.000	0.309	0.172	1.836	1.945	0.010 .
	MAT	0.000	0.309	0.172	0.013	0.415	0.693
	Non-C_4: MAT	0.000	0.309	0.172	-0.080	-1.755	0.130
14	C_4	0.000	0.352	0.145	-0.155	-0.178	0.865
	Non-C_4	0.000	0.352	0.145	1.766	1.441	0.200
	MAT	0.000	0.352	0.145	-0.009	-0.212	0.840
	Non-C_4: MAT	0.000	0.352	0.145	-0.071	-1.189	0.279

Note: λ refers to Pagel's estimator of phylogenetics. Significance level: · , $p < 0.1$; *, $p < 0.05$; **, $p < 0.01$. Abbreviation: DAC; Day after chilling.

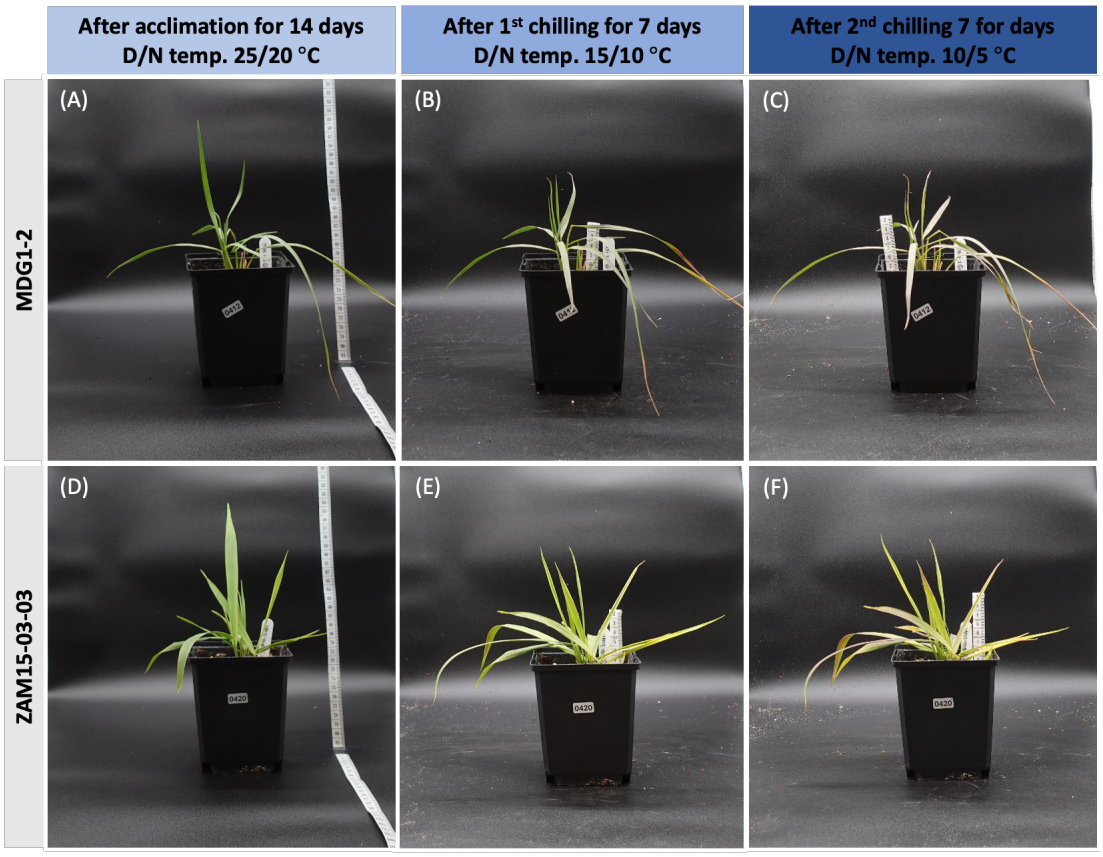

Figure S5.3 Two genotypes of *A. semialata*, MDG1-2 (A-C) and ZAM15-03-03 (D-F), show the alterations in leaf phenotypes such as leaf tip necrosis and red colouration after exposure to a two-phase decrement of chilling temperatures for 14 days. Abbreviation.: D/N temp. = day/night temperature.

Chapter 6

General Discussion and conclusions

1. How C_4 grasses tolerate low temperatures

The grass family is one of the plants that has successfully migrated, diversified and survived under low temperatures (Hopkins et al., 2006; Linder et al., 2018). To tolerate low temperatures, some grasses have evolved anatomical traits to adapt to survive under suboptimal conditions, with associated modifications in the biochemistry and physiology of these plants. Since these changes are inherited, they represent cold adaptation. However, some plants can thrive and survive under low temperatures without these constitutive adaptations. They can acclimate to low temperatures by temporarily altering their biochemical processes, which leads to the maintenance and compensation of physiological performance. This phenomenon is called cold acclimation. In our study, we found both adaptations and acclimation to low temperatures in the grass species, which are explained as follows (Figure 6.1).

1.1 Cold adaptations in C_4 grasses

Plants may adapt to low temperatures through developmental changes in several parts of the plant, including leaves, stems, and roots. However, our study focused only on leaves since they are the parts of plants where photosynthesis occurs. We compared leaf anatomical adaptations between temperate and tropical grass species, as well as intraspecific adaptations among different *Alloteropsis semialata* genotypes with varying geographical origins. In our study, we observed the four major leaf anatomical adaptations to low temperatures, as follows.

1.1.1 Bundle sheath (BS) size

Kubien and Sage (2004) suggested that C_4 grasses might enlarge their RubisCo-containing cells (i.e., BS cells) to sustain photosynthetic efficiency, as RubisCo becomes the rate-limiting enzyme for C_4 photosynthesis under low temperatures. However, our findings in Chapter 2 show no statistically significant difference in BS area between tropical and temperate grasses. This lack of statistical significance may be due to the limited number of biological replications or the narrow range of species

examined in our study. Similarly, our investigation of intraspecific leaf adaptations in *A. semialata* (Chapter 3) found no correlation between BS area and geographical provenance (mean annual temperature; MAT). These results align with Pignon *et al.* (2019), who found no evidence of enlarged BS chloroplast volume per leaf area in chilling-tolerant species compared to chilling-sensitive ones, suggesting that BS enlargement is not necessarily related to RubisCo content. Additionally, Naidu and Long (2004) highlighted that RubisCo is not the sole limitation for C_4 photosynthesis under chilling conditions. Multiple factors, such as damage to photosystem I or II (Kudoh et al., 2002; Wang et al., 2008) and restricted electron transport at low temperatures (Labate et al., 1989a), can also decrease photosynthesis. These studies indicate that multiple factors can influence BS size. Therefore, it is insufficient to attribute changes in BS size solely to RubisCo content. For a more comprehensive understanding of how BS cells adapt to low temperatures in grasses, future research should include a larger sample size, a greater variety of species, and an exploration of additional factors affecting BS cell size in grasses. This comprehensive approach will help uncover the various mechanisms that allow grasses to maintain photosynthetic efficiency in cold conditions.

1.1.2 Large phloem area in minor vein (MV)

Our study shows that *Alloteropsis* genotypes from colder areas have enlarged phloem area and greater cell numbers. We used a mathematical model in Chapter 3 to predict phloem flow rate in *Alloteropsis* based on information from other species, and found that the flow rate may decrease by 80% when the temperature drops from 25 to 15 °C. However, our findings show that the combined effects of an increase in cell number and cell radius of phloem may increase the flow rate up to seven times, which would be more than sufficient to compensate for the flow rate that is lost due to low-temperature impacts. Altogether, these adaptations imply structural changes to compensate for the impairment of sugar loading under low temperatures and are essential for a plant to maintain or continue its growth and metabolism processes like photosynthesis under suboptimal conditions. However, studies about phloem adaptation and sugar translocation in grasses are still limited and need more exploration.

1.1.3 Small metaxylem in major veins

Although area and mean cell size of minor vein xylem do not differ between temperate and tropical grasses, our findings show that temperate grasses have evolved smaller metaxylem vessels in major veins. This result is consistent with several studies indicating that plants experiencing sub-zero temperatures, such as grasses inhabiting temperate, alpine, and high-altitude regions, exhibit reductions in metaxylem vessel size, as observed in barley (*Hordeum vulgare*) (Tamang *et al.*, 2022) and Bermuda grass (*Cynodon dactylon*) (Ahmad et al., 2016). Smaller metaxylem vessels are associated with preventing bubble formation or embolism in xylem vessels at sub-zero temperatures (Ahmad et al., 2016), thus helping to maintain water hydraulic conductance and transport from root to leaf. Additionally, Brodribb and Field (2000) found a positive correlation between xylem hydraulic conductance and photosynthetic efficiency. This suggests that having smaller xylem vessels is one of the adaptations that help grasses tolerate low temperatures, especially those experiencing sub-zero environments. However, despite the diverse geographical origins of *A. semialata*, we did not observe the adaptation in this species. This could be explained by the mean annual temperature (MAT) among different genotypes, which ranges from 15 to 25°C (Fick et al., 2017), indicating that they do not typically experience sub-zero temperatures in their natural habitat. Thus, this adaptation may not be necessary for survival in nature, except at the range limit of this species (Osborne et al., 2008).

1.1.4 Low leaf moisture content

The previous study by Watcharamongkol (2019) found that temperate grasses exhibit lower leaf moisture content compared to tropical ones. Our findings find an anatomical explanation for this observation, as we discovered a positive correlation between bulliform cells, epidermis, and mesophyll cell sizes with leaf moisture content. This suggests that temperate grasses have evolved smaller cell sizes in these tissues with an associated drop in water content to survive under low temperatures. Bulliform cells are large epidermal cells containing water and are involved in leaf rolling and expansion by regulating water intake (Metcalfe, 1960; Ellis, 1976). Therefore, alterations in water content in both bulliform cells and epidermis may affect leaf moisture content. Regarding mesophyll cells, this relationship is consistent with a

previous study by Garnier and Laurent (1994) which stated that leaf moisture content depends on the proportion of mesophyll in the leaf cross-sectional area.

Altogether, we observed that leaf anatomical adaptations to low temperatures are generally consistent across C_4 grasses. However, it is important to note that we did not observe certain adaptations in *A. semialata*, which has not encountered freezing temperatures widely in its natural habitat. Moreover, this implies that these adaptations may develop later after diversification into cooler climates.

1.2 Cold acclimation in C_4 grasses

As mentioned, acclimation to low temperatures results from alterations in biochemistry, resulting in the maintenance of plant physiology, involving various processes. From our study, we categorise these into three main processes as follows.

1.2.1 Maintaining photosynthetic efficiency

One of the most critical aspects of plant adaptation to low temperatures is their ability to maintain and compensate for lowered photosynthetic efficiency. Previous studies on cold-tolerant grasses have indicated an increase in photosynthetic enzyme contents and gene expression in response to low temperatures. For instance, research on *Miscanthus x giganteus*, a particularly cold-tolerant C_4 grass, has shown consistent chlorophyll fluorescence parameters indicative of unchanged PSII photochemistry even after exposure to low temperatures (Long and Spence, 2013; Bilska-Kos *et al.*, 2018). However, our findings are not consistent with these studies. We observed a continued decrease in photosystem II (PSII) efficiency across all genotypes of *A. semialata*, both C_4 and non-C_4, after exposure to chilling conditions with high-light intensity for 14 days. Furthermore, our transcriptomic analysis revealed a decrease in the expression of genes encoding RubisCo and PEPC in all three grasses in our study, including both C_4 tropical and C_3 temperate grasses. This suggests a limited ability of these grasses to maintain photosynthetic efficiency at low temperatures, indicating their vulnerability to prolonged cold conditions. Additionally, chlorophyll, a crucial pigment for photosynthesis, exhibited varying responses among different *Alloteropsis* genotypes, particularly in non-C_4 grasses, suggesting differential abilities to maintain chlorophyll

content under chilling conditions. Interestingly, the C_3 temperate grass in our transcriptomic study uniquely expressed increased biosynthesis of phylloquinone (vitamin K1) and chloroplast differentiation compared to the C_4 tropical grasses. However, both C_3 and C_4 grasses exhibited inhibition of cellular and chloroplast gene expressions, indicating shared impacts on plant growth and development in response to low temperatures. These findings suggest that C_4 grasses may have insufficient mechanisms to maintain chlorophyll content and chloroplast functions under chilling conditions compared to C_3 counterparts.

Furthermore, the NPQ mechanism, a common response in *A. semialata*, was observed to increase over time in all genotypes with different responding degrees, indicating its role in protecting plants from photoinhibition and maintaining photosynthesis under chilling conditions. Additionally, our results demonstrated the downregulation of genes involved in reactive oxygen species (ROS) scavenging in both C_3 temperate and C_4 tropical grasses, suggesting a reduction in their ability to deal with photooxidative stress at low temperatures. While C_3 and C_4 grasses exhibited similar responses in terms of gene expression, our findings also suggest variations in ROS scavenging mechanisms among these grasses, with potential implications for maintaining cellular homeostasis under cold conditions.

Finally, another response to reduce photooxidative stress is the accumulation of anthocyanin in plant leaves, enhancing antioxidant capacities to reduce oxidative stress induced by low temperatures or high-intensity light (Chalker-Scott, 1999; Sivankalyani et al., 2016). We observed anthocyanin accumulation in some genotypes of *A. semialata* after exposure to chilling with high-light conditions for 14 days. This response is commonly found in various grasses, such as *Zoysia japonica* thriving under low-temperature conditions (Jin et al., 2022), and rough bluegrass (*Poa trivialis L.*) responding to high-intensity light (Petrella et al., 2016).

1.2.2 Maintaining transportation of water and sugar

Our transcriptomic analysis indicates that during exposure to low temperatures, two genes, PTR2 and MSSP2, involved in cellular peptide and sugar transportation respectively, are upregulated in both C_4 tropical species but downregulated in C_3 temperate species. PTR2, or Peptide Transporters 2, facilitates the transport of various peptide types (Choi *et al.*, 2020), while MSSP2 encodes a monosaccharide-sensing protein 2 responsible for sugar transport to the tonoplast or vacuole (Wormit *et al.*, 2006; Schulz *et al.*, 2011). This suggests that C_4 tropical species respond to low temperatures by increasing peptide and monosaccharide transport, which are subsequently stored in the vacuole rather than being transported into the phloem. Conversely, C_3 temperate species may exhibit a different response or potentially employ alternative mechanisms.

1.2.3 Maintaining cellular metabolism

Grasses have developed notable acclimation to low temperatures, involving complex cellular processes found in both plants with C_3 and C_4 photosynthesis pathways. Our transcriptomic analyses reveal the expression of genes responsible for producing essential metabolites and energy needed to maintain cellular functions during chilling conditions found in the C_3 temperate grass, suggesting that essential metabolism can be maintained, and it may need energy to compensate for various metabolic and physiological processes that may be impaired under low temperatures. Moreover, our results also show that active ion transmembrane transporters play a crucial role in responding to low temperatures in C_4 grasses. Although the downstream mechanisms of certain genes, like Ca^{2+} channels and the plasma membrane H^+-ATPase, remain unclear in grasses, they are known to be vital for cold adaptation in various plant species to maintain various physiological functions such as intracellular pH regulation, stomatal opening, nutrient uptake, and cell growth (Janská *et al.*, 2010). Interestingly, genes involved in these active ion transporters are also found in the C_3 counterparts, indicating shared strategies between C_3 and C_4 pathways to cope with the impacts of cold.

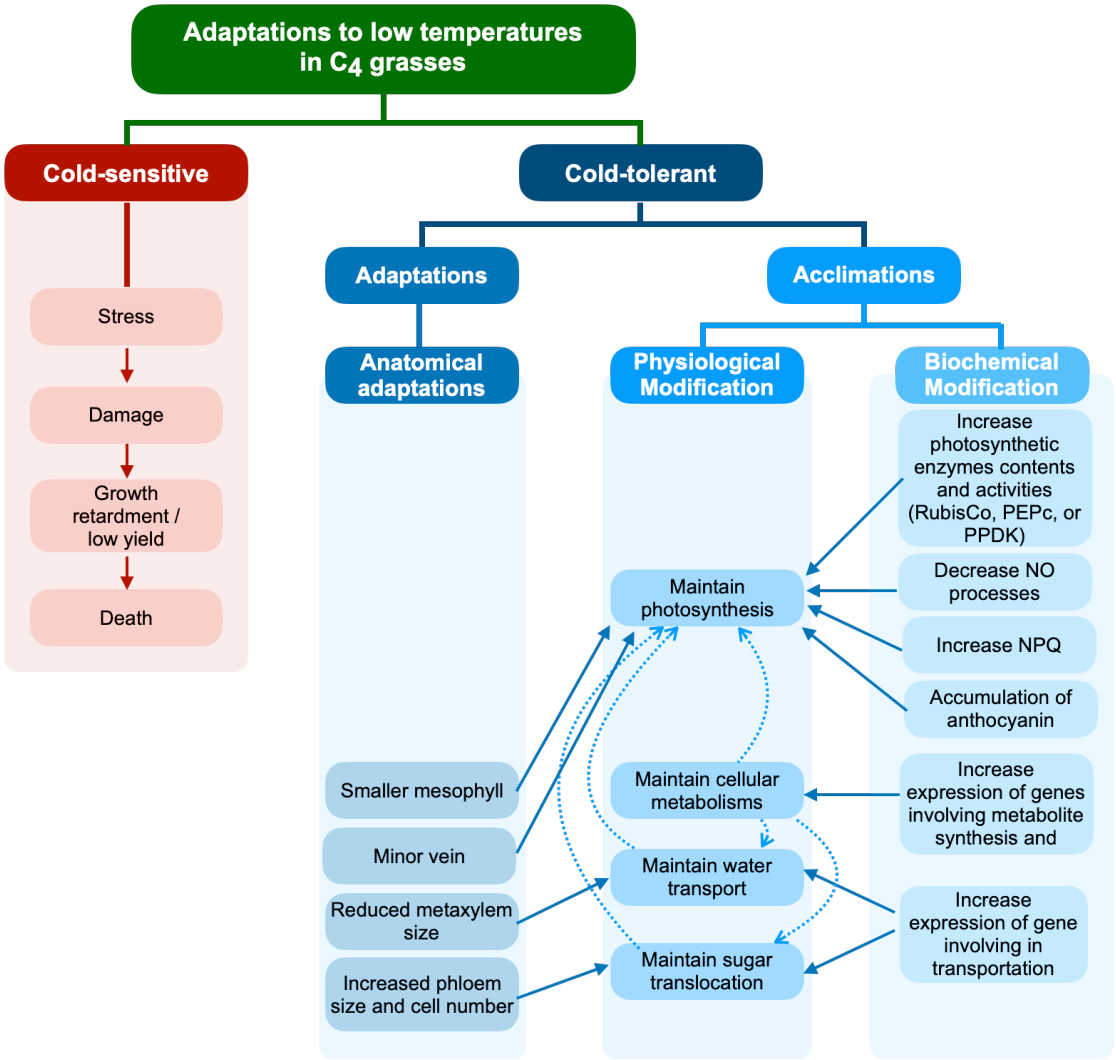

Figure 6.1 Summary of adaptations to low temperatures in C_4 grasses based on our study and literature review.

2. Impacts of high-light intensity and photodamage in cold-acclimated grasses

Some grasses exhibit the ability to acclimate and thrive under low-temperature conditions, but the impact of light intensity stands as another crucial factor affecting grasses independently of temperature. As temperatures drop, biochemical processes, particularly photosynthesis, slow down, leading to a reduction in the utilisation of light energy for photochemical processes and subsequent excess energy dissipation. Without sufficient quenching mechanisms in place, or if existing mechanisms are insufficient, plants may experience photooxidative stress, resulting in photoinhibition and photodamage.

Our investigation into physiological alterations in *A. semialata* under high-light intensity with two phases of chilling treatments over 14 days revealed noteworthy findings. During the initial phase of chilling with a day/night temperature of 15/10 °C, the energy passing through PSII and NO processes exhibited a slight decrease day by day, with varying degrees of response among different genotypes. Conversely, NPQ increased over time. Throughout this chilling phase, some genotypes displayed physical signs of photodamage such as leaf yellowing, chlorosis, and wilting. However, by the end of the second phase of chilling treatment with a day/night temperature of 10/5 °C (DAC14), certain genotypes exhibited more signs of damage, including necrosis, growth retardation, and death. Taken together, these observations suggest that temperatures below 10 °C are unsuitable for the growth and survival of *A. semialata*. Furthermore, they underscore the importance of maintaining photosynthesis by dissipating excess energy via NPQ mechanisms below this suboptimal temperature.

Interestingly, we discovered a correlation between the C_4 photosynthetic pathway and alterations in photosynthetic parameters, characterised by a decrease in Phi2 and PhiNO and an increase in PhiNPQ on the last day of the experiment (DAC14). However, no statistical significance was observed in the non-C_4 pathway. This finding suggests that C_4 genotypes may employ distinct regulatory mechanisms to maintain photosynthesis, unlike their non-C_4 counterparts. In a study by Ripley *et al.* (2007), although *A. semialata* under drought conditions exhibited a decrease in PSII efficiency

in both C_3 and C_4 genotypes, the critical role of NPQ mechanisms in dissipating excess energy as heat and maintaining photosynthetic balance was emphasised specifically in C_4 plants. Therefore, it is conceivable that C_4 genotypes utilise this mechanism to sustain their photosynthesis.

3. Evolution in grasses adapting to low temperatures

The grass is a plant family that has evolved diverse mechanisms to tolerate low temperatures, ensuring its survival in varied climatic conditions. Through complex physiological and biochemical adaptations, grasses exhibit both common and unique responses to cold, reflecting their evolutionary history and ecological niches.

One of the fundamental processes affected by low temperatures is photosynthesis, which sustains plant growth and development. Our transcriptomic study reveals that both C_3 temperate and C_4 tropical grasses experience reductions in photosynthetic efficiency under chilling conditions. Common responses include the reduction of ROS scavenging, induction of photoinhibition in PSII, and inhibition of cellular and chloroplast gene expressions. Furthermore, our results show that the genes encoding for RubisCo and PEPC were downregulated in both C_3 temperate and C_4 tropical grasses, which may lead to a low rate of photosynthesis. Altogether, this suggests that these negative impacts suppress plant growth and development and underscore the vulnerability of grasses to low temperatures.

Notably, our findings from the transcriptomic study show that the C_3 temperate grass has evolved mechanisms to maintain photosynthesis through the biosynthesis of phylloquinone (vitamin K1) and increased chloroplast differentiation. In contrast, C_4 tropical grasses exhibit increased expression of genes involved in peptide and sugar transport for storage in vacuoles, a response that was downregulated in the C_3 grass. This divergence in response mechanisms reflects the unique evolution of these grasses and their adaptation to different environmental conditions.

Furthermore, our study highlights the importance of cellular metabolism in cold tolerance mechanisms. Genes involved in generating precursor metabolites and energy, as well as active ion transmembrane transporters, are upregulated in response to low

temperatures in the C_4 grasses. These responses were observed in the C_3 grasses as well, however we did not access their expression levels. These molecular responses indicate the complex interplay between cellular processes and environmental factors, enabling grasses to maintain cellular homeostasis and physiological functions under low temperatures.

In conclusion, our study reveals that grasses have evolved diverse strategies to cope with low temperatures, reflecting their evolutionary history and ecological niches. While common responses such as reductions in photosynthetic efficiency are observed across C_3 and C_4 grasses, unique adaptations, such as phylloquinone biosynthesis in C_3 temperate grasses and enhanced peptide and sugar transport in C_4 tropical grasses, highlight the versatility of grasses in responding to environmental challenges.

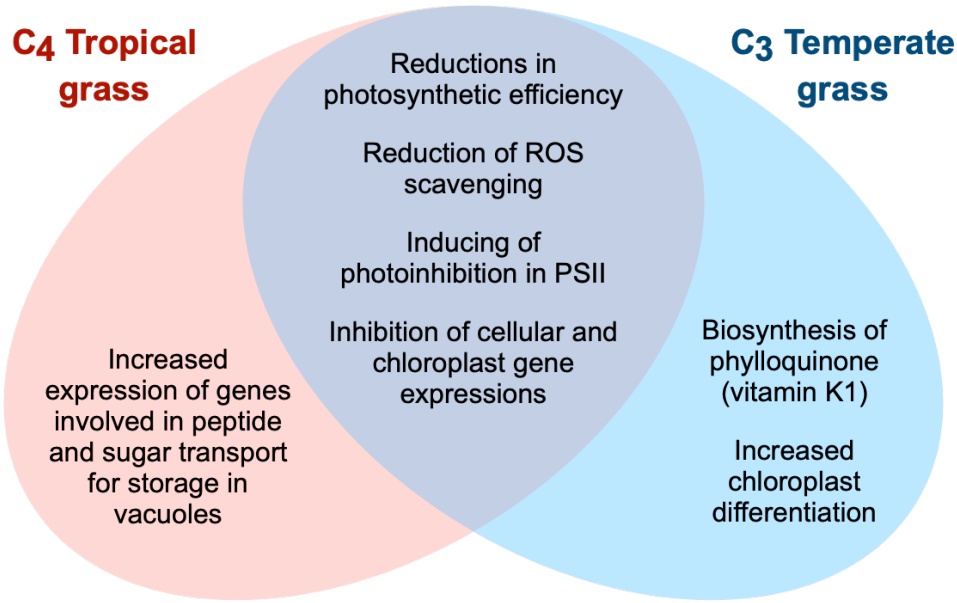

Figure 6.2 The comparison of adaptations and responses to low temperatures between C_3 and C_4 grasses based on our study

4. Future directions

The primary objective of our study is to elucidate the mechanisms enabling C_4 grasses to thrive under low-temperature conditions, with implications for future food security. Understanding the evolutionary adaptations of grasses to low temperatures not only sheds light on their ecological success but also informs efforts to enhance their resilience in the face of climate change. While we have contributed novel insights into how C_4 grasses adapt to cold through leaf anatomical changes and acclimation responses, our study has been constrained by time and cost limitations, preventing a comprehensive exploration of all relevant factors. To enhance and broaden our understanding in this area, we propose various suggestions for future research.

Regarding the study of leaf anatomical adaptation, a key limitation lies in the resolution of the light microscope, which hindered the precise distinguishing of individual cell borders, cell types, and measurement of cell wall thickness, particularly in small cells like the xylem and phloem vessels within minor veins. To address this limitation, we recommend using electron microscopy to accurately measure alterations in cell wall thickness.

We have made an interesting observation regarding the enlargement of the minor vein phloem area in the leaves of *A. semialata* from colder regions. However, existing literature on how grasses adapt to low temperatures in this manner is limited. We propose measuring the rate of phloem flow to verify whether the increased area and number of phloem vessels are indeed linked to compensation for phloem flow at low temperatures. Measuring the rate of phloem flow in grasses can be more challenging than in woody plants due to their smaller size and different anatomical structures. However, several techniques can be adapted for use in grasses such as aphid stylet technique, radioactive isotope labelling, and fluorescent dye tracing. Additionally, considering factors such as the 3D structure of sieve cells, including pore distribution on both sides of sieve and sieve plates, could provide further insights into this adaptation mechanism. Various advanced microscopy techniques can be used to observe the 3D structure of cells such as confocal laser scanning microscopy, scanning electron microscopy (SEM), and X-ray microcomputed tomography (Micro-CT).

Our finding of smaller major vein metaxylem in temperate grasses compared to tropical ones suggests a potential role in maintaining water transport at low temperatures. To confirm this hypothesis, we recommend measuring hydraulic conductance in plants exposed to low temperatures, including freezing conditions or below. There are various ways to measure hydraulic conductance in grasses such as using pressure chamber, flow meter, or xylem flow sensor. Furthermore, we suggest investigating the minor vein xylem, as it may also play a crucial role in plant adaptation to cold, despite being overlooked in our study due to microscope resolution limitations.

While we observed various physical alterations in *Alloteropsis* leaves exposed to chilling with high-light intensity, our measurement of leaf greenness using the SPAD index provides only indirect insight. To obtain more accurate pigment content measurements, we recommend using chemical methods such as spectrophotometry or high-performance liquid chromatography (HPLC) to assess pigment content accurately, including chlorophyll, carotenoids, and anthocyanins.

In our study, we did not measure the contents and activities of photosynthetic enzymes such as RubisCo and PEPC. However, incorporating these measurements would be essential to confirm the findings of our transcriptomics study and provide a more comprehensive understanding of the underlying mechanisms of photosynthetic adaptation to low temperatures.

In summary, our research reveals a number of ways that C_4 grasses cope with cold climates. We discovered unique and consistent leaf anatomical changes in temperate C_4 grasses, and we observed similarities and differences in gene expression between C_3 and C_4 species. These findings emphasise how both the type of photosynthesis and geographical provenance influence the ability of grasses to tolerate low temperatures. By overcoming current limitations and exploring future research paths, we can better understand the complex mechanisms behind their ability to withstand suboptimal environments.

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