Exploring the role of boron and cell wall crosslinking in plant freezing tolerance and stomatal development

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ABSTRACT

Abiotic stresses such as freezing and drought can be extremely harmful to plants, resulting in substantial yield losses or even death. Freezing events can cause freezing-induced dehydration, therefore, many of the pathways involved in freezing tolerance are also involved in drought tolerance. The cell wall is a complex polysaccharide layer which has many essential roles in plant growth, defence and survival. Several studies have linked the cell wall to freezing tolerance. In addition, cell wall pectin rhamanogalacturonan-II (RGII) has recently been implicated in freezing tolerance.

The role of RGII dimerisation in freezing tolerance was investigated using mutants with altered dimerisation. *mur1* mutants show reduced RGII dimerisation as a result of decreased levels of L-fucose while *bor* mutants show reduced dimerisation due to defective boron transport. Both RGII dimerisation mutants show reduced freezing tolerance indicating that dimerisation plays a role in freezing tolerance. This is supported by the fact that freezing tolerance was restored upon restoration of RGII dimerisation via supplementation.

Dimerisation mutants were seen to have increased desiccation and transpiration which improved with restoration of RGII dimerisation. Other factors may be involved in this response, however, RGII dimerisation mutants displayed an altered stomatal phenotype with a missing cuticular ledge. Again, supplementation with boron was able to restore a WT-like stomatal phenotype. Alterations to the stomatal morphology may be the cause of altered transpiration in these mutants as stomata are essential for control of gas exchange and mediation of water loss. However, further research is required to determine the exact role of RGII dimerisation in the desiccation response.

These findings highlight the importance of both the cell wall and RGII in drought and freezing tolerance. This research could identify new areas of interest for development of crop lines with improved freezing or drought resistant.

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Standard scientific abbreviations are used for units of amount, length, molarity, temperature, time and weight. Standard symbols are used for chemical elements.

Standard scientific conventions have been used for protein and gene naming: wild type genes and proteins are capitalised while mutant names are italicised and lowercase and gene names italicised and capitalised.

ABA	Abscisic acid
AIR	Alcohol insoluble residue
BA	Boric acid
CBF	C-repeat/drought responsive element binding factor
COR	Cold regulated
EL	Electrolyte leakage
EPG	Endo-polygalacturonase
GalA	Galacturonic acid
HG	Homogalacturonan
LSM	Least squared means
ML	Middle lamella
MS	Murashige and Skoog
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PCW	Primary cell wall
PG	Polygalacturonase
RGI	Rhamanogalacturonan- I
RGII	Rhamanogalacturonan- II
ROS	Reactive oxygen species
SCW	Secondary cell wall
SEM	Scanning electron microscopy
SFR	Sensitive to freezing
T-DNA	Transfer DNA
TEM	Transmission electron microscopy
WT	Wild type

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CHAPTER 1

INTRODUCTION

Due to their sessile nature, plants must adapt to the stress they encounter in their environment in order to survive. A major stress faced by plants is temperature fluctuation which can negatively impact crop yields (Tack, Barkley and Nalley, 2015). Temperature fluctuation can lead to both freezing and drought events which can be very damaging to crop plants and their yields (Lardon and Triboi-Blondel, 1995; Chuang Zhao *et al.*, 2016).

Freezing is particularly harmful to plants as it can result in serious damage that can lead to crop loss and, in more extreme cases, death (Levitt, 1980). There is a huge amount of variation in how plants respond to freezing: tropical plants are poorly adapted to survive cold and can show chilling damage at temperatures as high as 10-12°C (Lyons, 1973). In comparison some plants, particularly those that grow in temperate regions, have adapted to be more tolerant to freezing and protect themselves against this damage (Burke *et al.*, 1976). The importance of understanding this ability to protect against freezing damage becomes ever more apparent in the face of climate change and increasingly unpredictable weather. Climate change brings early and late frosts which can be very damaging to unprepared plants. Early autumn or late spring frosts can be particularly harmful to plants as they occur either before tissues have hardened to prepare for winter or after they have de-hardened (Malmqvist, Wallertz and Johansson, 2018; Meier, Fuhrer and Holzkämper, 2018).

Freezing induced dehydration means that many of the mechanisms involved in freezing tolerance are also involved in drought tolerance (Chen, Li and Burke, 1977; Willemot and Pelletier, 1979). Drought is a very serious stress faced by plants globally, it can affect everything from germination to growth and yields (Chowdhury *et al.*, 2016; Nadeem *et al.*, 2019). While some plants show a degree of drought tolerance the extent of the damage caused is highly dependent on the developmental stage of the plant as well as the intensity and duration of the drought (Nadeem *et al.*, 2019). Drought is an important limiting factor in crop production and the increase in temperature predicted to occur as a result of global warming presents a major cause for concern.

As the population continues to grow, food security becomes an increasingly pressing issue. Understanding why some plants show innate freezing or drought tolerance could aid the development of crop strains that can survive unfavourable weather conditions; this would reduce plant losses and yield reductions that occur due to unfavourable conditions. Ultimately this has the potential to expand the regions in which certain crop plants can be grown to

include areas with more varied or unfavourable weather conditions (Sanghera *et al.*, 2011). However, traditional selective breeding methods to improve crop freezing tolerance have been attempted with little success (Thomashow, 1999). Similarly, while some drought resistant crops have been developed through traditional breeding methods it is a time consuming and labour intensive process (Hussain *et al.*, 2018). A more in depth understanding into the mechanisms underpinning freezing and drought tolerance is required if we hope to utilize the innate ability of some plants to survive these unfavourable conditions.

This study investigates the role of the cell wall in freezing and drought tolerance, focusing specifically on the effects boron and cell wall crosslinking have in these responses.

1.1 Freezing

Freezing damage is usually caused by the formation and growth of ice crystals within the plant. Ice formation only happens spontaneously at highly supercooled temperatures, below -40°C, (Bigg, 1953) when water molecules come together to form a stable ice nucleus. This becomes increasingly likely as the temperature decreases. However, ice nucleation can occur at higher temperatures if catalysed by the presence of certain molecules or ice-nucleating bacteria (Lindow, 1983). It has also been suggested that certain sites associated with the cell wall may act as nucleation points (Salt and Kaku, 1967). When plants cannot avoid nucleation and ice growth, they freeze (Pearce, 2001).

Ice formed on the outer surface of the leaf can enter the plant via the stomata. As a protective mechanism, stomata close at night when temperatures are lower and freezing is more likely (Wisniewski and Fuller, 1999). This is thought to help plants avoid freezing, however, ice can also enter the leaf via hydathodes or cracks in the cuticle (Wisniewski and Fuller, 1999; Pearce, 2001). Alternatively, ice can form inside the cells in either the intracellular space or the extracellular space depending on freezing conditions (Mazur, 1963; Yamada *et al.*, 2002). Freezing can affect plants very differently depending on the rate at which the temperature decreases, the amount of water found both within the cell and in the surrounding tissues and the hardiness of the plant. These factors also affect where the ice is formed, the amount of ice formed and the damage the plant sustains (Asahina, 1956; Thomashow, 1999; Smallwood and Bowles, 2002).

1.1.1 Extracellular ice

Formation of extracellular ice in the apoplast is a common occurrence in nature and is responsible for most of the freezing damage seen in plants. Ice mainly forms between cells where there is space for growth and spreads from nucleation sites associated with the cell wall (Salt and Kaku, 1967; Levitt, 1980). Ice formation initially occurs in vessels where the solute

concentration is lower before spreading to epidermal cells (Asahina, 1956). At low cooling rates the hydrophobic lipid membrane protects the cell by preventing ice growth into the cell; this can protect tissue that is more susceptible to freezing or damage (Chambers and Hale, 1932; Smallwood and Bowles, 2002).

Extracellular ice formation occurs in a two-step process: the initial formation of ice alters the water potential gradient causing intercellular water to move from the cytoplasm to the apoplast, melting the ice. This is followed by more intense freezing in which ice crystals use intercellular water released into the apoplast to grow and expand (Pearce and Fuller, 2001). This decrease in intercellular water can be very detrimental to cells and can lead to freezing induced dehydration (Gusta, Trischuk and Weiser, 2005).

1.1.2 Intracellular ice

Intercellular ice forms in the symplast, vacuole or cytoplasm and is an unusual occurrence in nature. As previously stated, extracellular ice draws water out of the cell, this causes an increased intracellular solute concentration. The nucleation point of ice is decreased when water has a high solute concentration therefore, as the solute concentration within the cell increases it diminishes the likelihood of intracellular freezing (Levitt, 1980; Pearce, 2001). As a result very fast cooling is normally required for intracellular ice formation to occur (Mazur, 1963; Levitt, 1980). Thus, the formation of intracellular ice is a far rarer occurrence in nature than extracellular ice (Weiser, 1970).

However, the permeability of the cell membrane can also influence where and when ice formation occurs. If the membrane has very limited permeability water movement out of the cell will be slow. This means the cell solute concentration will not be as high, which allows intracellular freezing to occur at a lower temperatures (Levitt, 1980).

1.1.3 Freezing Damage

Freezing can cause many different types of damage depending on the location and rate at which ice formation occurs. Protein denaturation can occur as cold can affect the conformational stability of proteins, which in turn affects processes within the cell (Guy, Haskell & Li 1998). Freezing can also result in the formation of oxygen radicals which can cause membrane and organelle damage (Kendall and McKersie, 1989). However, it is thought that cell membranes are the main site of freezing damage. Ice can separate the epidermis from other tissues (Pearce, 2001) and large pieces of ice can lacerate the cell causing death (Levitt, 1980).

Freezing induced dehydration is considered to be the predominant cause of damage by extracellular ice (Levitt, 1980). Extracellular ice causes cellular water to be drawn across the

plasma membrane dehydrating the cell. Dehydration can lead to cellular concentration and collapse allowing cell to cell adhesion and fracture jump lesions to form resulting in damage or death (Pearce, 1988; Nagao *et al.*, 2008). Cellular collapse can also cause the plasma membrane to come into close contact with other cellular structures such as chloroplasts. This causes the phospholipids to undergo a mesomorphic phase transition from lamellar to hexagonal II phase, this can prevent the protoplast from returning to its original size and shape upon thawing (Steponkus, 1984).

Freeze-thaw cycles also cause high levels of cellular damage. Freezing induced dehydration causes cellular collapse; to maintain tension the cell membrane shrinks and excess lipids are removed and stored in endocytotic vesicles (Steponkus, 1984). When the ice thaws a large amount of water is released and attempts to re-enter the cell where the solute concentration is much higher. The sudden osmotic expansion causes the membrane to rupture as the lipids that were removed cannot be returned to the membrane quickly enough. This is known as expansion induced lysis (Wiest' And and Steponkus, 1978).

Some cells become resistant to cellular collapse as they increase freezing tolerance. This causes negative pressure inside the cell which can limit the amount of dehydration cells experience. However, negative pressure can cause cavitation. This occurs when a vapour bubble forms within the cell and can lead to rupture, damage or cellular collapse when the plant defrosts and normal pressure is restored (Rajashekar and Lafta, 1996a; Smallwood and Bowles, 2002). Resistance to cellular collapse is thought to be linked to cell wall strength. This is believed to increase during cold acclimation which is a process some plants utilise to improve their freezing tolerance.

1.2 Cold acclimation

Plants from tropical regions are unlikely to experience cold temperatures, therefore, they are not able to survive freezing. These plants sometimes display chilling damage at temperatures as high as 10-12ºC (Lyons, 1973). In contrast, temperate plants tend to be more resistant to freezing, although this tolerance can vary. In order to survive freezing events plants must adapt in response to their changing environment; they do this via a process known as cold-acclimation (Burke *et al.*, 1976).

Cold-acclimation occurs in response to a period of low, non-freezing temperatures (0-5°C) and is an important process that many temperate plants utilise to enhancing freezing tolerance and preventing freezing induced injury in plants (Levitt, 1980; Thomashow, 1999). There are many diverse mechanisms that plants utilise to protect against freezing even within the same species (Hannah *et al.*, 2006). However, exposure to non-freezing autumnal temperatures is

known to trigger biochemical and molecular changes that render the plant more tolerant to sub-zero temperatures. One important way in which plants respond to these environmental stimuli is by altering gene expression (Guy, Niemif and Brambl, 1985; Thomashow, 1999; Chinnusamy, Zhu and Zhu, 2007). However, in order to alter gene expression in response to cold, plants must first sense the temperature change.

1.2.1 Temperature sensing

Cold-acclimation is understood to trigger differential expression of more than 1,000 genes which improve the plants ability to survive freezing events (Guy, Niemif and Brambl, 1985; Thomashow, 1999; Smallwood and Bowles, 2002; Chinnusamy, Zhu and Zhu, 2007). However, the exact mechanism of cold perception in plants remains unknown. Changes in membrane fluidity and calcium (Ca^{2+}) are considered to be early signalling events that lead to changes in downstream targets (Knight and Knight, 2012). Changes in membrane fluidity can cause opening of Ca^{2+} channels and low temperature causes increases in cytosolic free Ca^{2+} (Knight *et al.*, 1991). Changes in Ca^{2+} concentrations trigger signalling cascades some of which impact gene expression in downstream cold responsive targets (Knight, Trewavas and Knight, 1996; Tähtiharju *et al.*, 1997; Saijo *et al.*, 2000; Guo, Liu and Chong, 2018). Kinases in the plasma membrane can also be activated in response to cold, such as responsive protein kinase 1 (CRPK1). CRPK1 transduces signals into the nucleus and may be involved in C-repeat/Drought Responsive Element Binding Factor (CBF) signalling (Liu *et al.*, 2017).

There are multiple pathways induced by cold-acclimation including activation of the CBF pathway and the transcription factors dependent on it (Fowler and Thomashow, 2002; Thomashow, 2010). This is the most well characterised cold-acclimation pathway and has an important role in freezing tolerance. The CBF pathway controls expression of more than 100 cold-regulated (*COR*) genes which are involved in cold acclimation and freezing tolerance (Jaglo-Ottosen *et al.*, 1998; Chunzhao Zhao *et al.*, 2016). This pathway is tightly regulated by many factors including Ca^{2+} concentration (Kurepin *et al.*, 2013; Shi, Ding and Yang, 2018). In addition, *COR* gene expression has been shown to be regulated by membrane fluidity, again indicating there may be multiple pathways involved in cold-induced gene expression (Ding, Shi and Yang, 2019).

1.2.2 Cold-acclimation targets

Expression of more than 1,000 genes are thought to be altered by cold-acclimation (Guy, Niemif and Brambl, 1985; Thomashow, 1999; Smallwood and Bowles, 2002; Chinnusamy, Zhu and Zhu, 2007). Decreased temperature causes changes in a wide range of cellular functions including photosynthesis, carbohydrate metabolism, polyamine synthesis, reactive oxygen

species (ROS) scavenging and protein folding (Fürtauer *et al.*, 2019). In addition, stabilising the cell structure and cell membrane integrity are also particularly important to prevent damage from freezing-induced dehydration (Thomashow, 1999). Alteration to lipid composition and sugar content can protect against damage such as freezing induced lesions and membrane fusion (Strauss and Hauser, 1986; Uemura, Joseph and Steponkus, 1995; Wanner and Junttila, 1999). Acclimation also causes differential expression of many genes involved in cell wall synthesis or remodelling resulting in structural changes to the cell wall in response to the period of cold (Weiser, Wallner and Waddell, 1990; Solecka, Zebrowski and Kacperska, 2008).

1.3 The Cell Wall

The cell wall is a highly complex polysaccharide layer and includes pectins, cellulose, hemicelluloses and proteins. It has many diverse and essential roles in plant survival ranging from ensuring the structural integrity and strength of the plant to controlling cell growth, differentiation and intercellular communication (Cosgrove, 2005). It is also the plant's first line of defence; acting as a protective barrier against pathogenic attack. In addition to this, it is becoming apparent that the cell wall plays a role in abiotic stress tolerance (Houston *et al.*, 2016). The cell wall is subject to remodelling in response to abiotic stresses such as pathogens, salinity, drought and frost which could form part of the stress acclimation process (Tenhaken, 2015; Houston *et al.*, 2016; Novaković *et al.*, 2018). It is possible that cold-acclimation in response to temperature decrease could also be part of this stress response process, as cold-acclimation has been shown to affect cell wall composition (Rajashekar and Lafta, 1996b; Solecka, Zebrowski and Kacperska, 2008). Cold acclimation is essential for plants to survive freezing events, however, as discussed, formation of ice crystals both on and in the cell wall during freezing can cause potentially irreversible damage (Pearce, 2001).

A plant cell wall is always composed of the primary cell wall (PCW) and middle lamella (ML). In addition, some cells, typically those that require structural reinforcement have an additional layer, the secondary cell wall (SCW). The PCW is composed of a pectin and hemicellulosic polysaccharide matrix containing proteins and a network of cellulose microfibrils. In comparison, the ML is primarily made of pectins and allows the formation of a continuous layer between adjacent cells (Brett and Waldron, 1996). Wall loosening must occur in the cell wall to enable growth and expansion. This is mediated by enzymes and changes in pH and wall deposition occurs in tandem to ensure the wall doesn't become thin (Cosgrove, 2016).

1.3.1 Cellulose

Cellulose is an important component of most cell walls. Interactions with other cell wall polysaccharides may be facilitated by cellulose-cellulose interactions (Cosgrove, 2018). Microfibrils can be formed when 30-100 cellulose chains bind together. These may affect the

direction of cellular growth (McN eil *et al.*, 1984) and can also form crystalline structures that are extremely important for cell wall strength (Cosgrove, 2005). In addition, hemicellulose, a type of polysaccharide, can cross-link microfibrils further strengthening the cell wall. However, it has been shown that microfibril movement is needed to facilitate cell wall growth (Zhang *et al.*, 2017). Xyloglucan is an important hemicellulose in the plant cell wall formed from a β-1,4-linked glucose residue backbone with various D-xylose, D-galactose and L-fucose residues attached (Hayashi, 1989). Variation in the sugar side chains attached to the xyloglucan backbone alters its confirmation and therefore, can impact its ability to form cellulose crosslinks and can influence the strength of the crosslinks formed (Hayashi, Marsden and Delmer, 1987; Levy, Maclachlan and Staehelin, 1997).

1.3.2 Pectin

Pectic polysaccharides are present in all layers of the cell wall and form a large portion of the ML. They are important for many physiological processes in the plant such as cell growth and differentiation (Voragen *et al.*, 2009). Pectins and hemicelluloses form the matrix component of the cell wall and help determine its rigidity and integrity. Pectins can also link together via covalent bonds to form complex structures in the cell wall (Brett and Waldron, 1996; Caffall and Mohnen, 2009a).

The plant cell wall contains many pectin polysaccharides such as arabinans, galactans and arabinogalactans, however, it is largely composed of homogalacturonan (HG) and rhamonogalactaronan-I and -II (RGI and RGII). Pectins are rich in galacturonic acid (GalA) residues which form chains with various branching side chains that differ between pectins. HG accounts for approximately 65% of pectin in the cell wall (Caffall and Mohnen, 2009b). It can form chains of up to 100 GalA residues which can be modified to alter function by the addition or removal of side chains of methyl and *O*-acetyl esters via enzyme activity (Daas *et al.*, 2001; Yapo *et al.*, 2007). RGI also accounts for a large proportion of cell wall pectin making up approximately 20-35% (Zablackis *et al.*, 1995). Finally, approximately 10% of cell wall pectin is made up of RGII (O'Neill *et al.*, 2004). To establish a better understanding of the cell wall mutants with alterations to their cell wall are often utilised.

1.4 Cell wall mutants

1.4.1 *mur1* and *sfr8*

murus1 (mur1) is a cell wall mutant which was identified in a screen investigating the roles of cell wall polysaccharides in plant growth and development (Reiter, Chapple and Somerville, 1993). *mur1* mutants were found to have only 2% of WT fucose levels in their shoot cell walls and 60% in their roots (Reiter, Chapple and Somerville, 1993). The *mur1* mutation was mapped to a gene encoding GMD2, a GDP-D-mannose-4,6-dehydratase, which is an enzyme involved in

the first step of GDP-L-fucose synthesis (Figure 1.1) (Bonin et al. 1997). *mur1* plants display dwarfism, reduced petiole and internode length and reduced apical dominance. However, it was possible to restore a WT-like appearance via supplementation with L-fucose (Reiter, Chapple and Somerville, 1993). *mur1* may also display reduced strength and stiffness of their cell walls as hypocotyl strength and force required to break inflorescence stems were seen to be reduced in these mutants (Reiter, Chapple and Somerville, 1993; Ryden *et al.*, 2003).

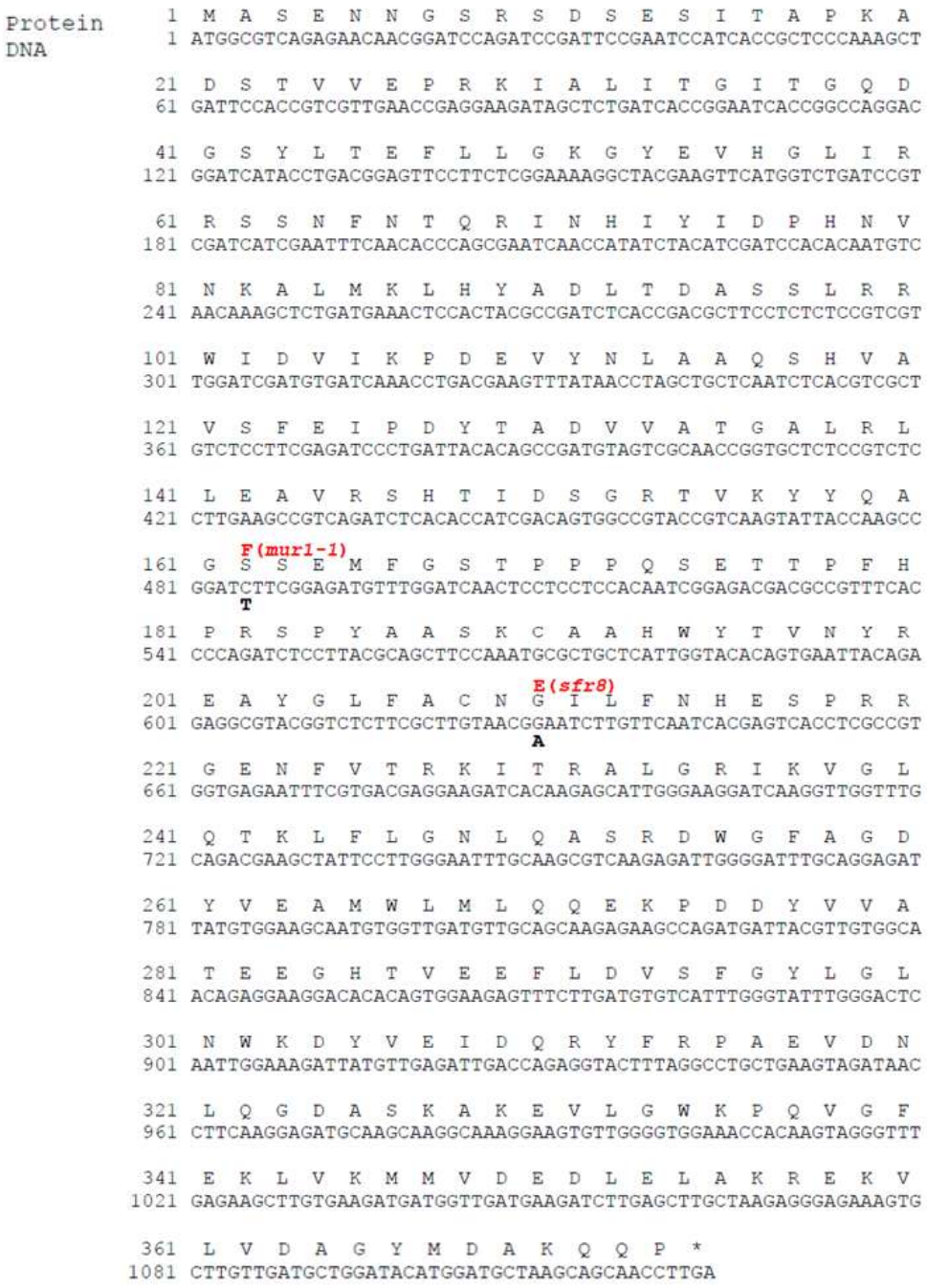

Figure 1.1: Nucleotide and amino acid sequence of *MUR1*. SNP and amino acid substitutions of the mutants *mur1-1* and *sfr8* are highlighted. Adapted from Panter *et al*. 2019

Sensitive-to-freezing-8 (*sfr8)* is a freezing-sensitive mutant identified in a screen for Arabidopsis mutants that were unable to cold acclimate to freezing conditions (Warren *et al.*, 1996). Positional cloning confirmed that *sfr8* has a mutation in the fucose biosynthetic gene *MUR1* and is an allele of *mur1*. s*fr8* has a single nucleotide polymorphism, G to A, that distinguishes it from WT (Figure 1.1).

sfr8 mutants displayed many of the phenotypes observed in *mur1* such as slightly shorter petioles and rounder smoother leaves than WT plants (Figure 1.2)(Gonçalves *et al.*, 2017). These mutants can also be seen to have slightly crumpled leaves, possibly caused by the shortened petioles. However, in some cases this phenotype can appear less pronounced leading to difficulty in differentiating between the two lines. Like *mur1,* the *sfr8* mutant also exhibits reduced ability to synthesise L-fucose and both *sfr8* and *mur1* show a marked reduction in freezing tolerance (Panter *et al.*, 2019).

Figure 1.2: Phenotypic assessment of WT and *sfr8* plants. Assessment of gross morphology in mature 5-week old Col-0 (WT) and *sfr8* plants.

Fucose is an important part of many cell wall components; it is found in glycoproteins, xylogucans, RGI and RGII. Mutants lacking fucose in their glycoproteins (Reiter, Chapple and Somerville, 1993) and xyloglucan (Zablackis *et al.*, 1995) both displayed a WT-like phenotype suggesting a lack of fucosylation in these cellular components is not responsible for the phenotypes displayed in *mur1* and *sfr8* plants. As such, it is suspected that the lack of RGII dimerisation in these mutants is responsible for the phenotypes and freezing sensitivity observed in *mur1* and *sfr8* mutants (Panter, 2019). However, the role that RGII dimerisation plays in the cell wall and freezing tolerance remains uncertain.

1.5 RGII and Boron

Rhamnogalacturonan-II (RGII) is a key cell wall pectin comprising of a homogalacturonan backbone of α-1,4-linked-DGalA residues and six sidechains, two of which have apiosyl sugar residues required for dimerisation (O'Neill *et al.*, 2001, 2004). Dimerisation of RGII monomers

is considered to be essential for plant growth and development as it supports cell adhesion and mechanical strength in the cell (Ryden *et al.*, 2003). RGII structure and dimerisation is highly conserved in plants (Matoh, Kawaguchi and Kobayashi, 1996) and it is thought that dimerisation occurs spontaneously (Ishii *et al.*, 1999). Boron is required for crosslinking RGII via a tetrahedral borate diol ester cross-link between apiosyl residues of the RGII subunits (Ishii and Matsunaga, 1996; Miwa *et al.*, 2010, 2013). It has been observed that this covalent bond always forms between the same adipose residues (side chain A) on both monomers (Figure 1.3)(Ishii *et al.*, 1999).

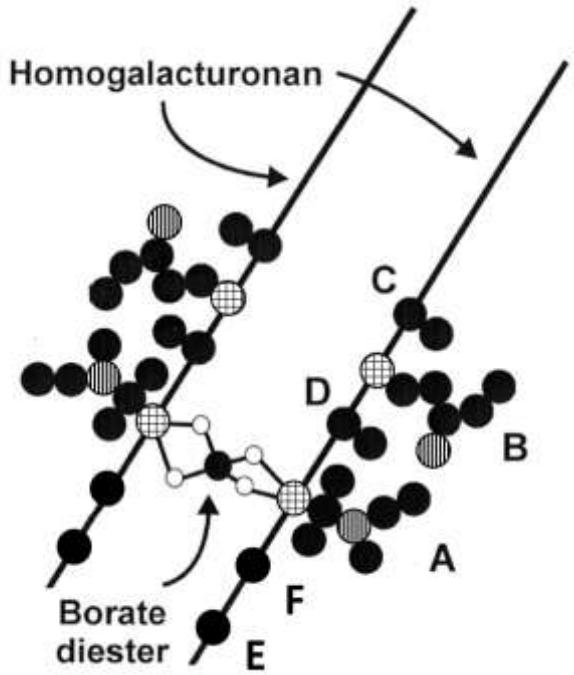

Figure 1.3: Structure of dimerised Rhamnogalacturonan-II (RGII). Homogalacturonan back bone and side chains A-F of each monomer are shown with L-fucose residues indicated in stripes. In addition, diester crosslinking between adipose residues (indicated by a hatched pattern) on side chain A can be seen (adapted from O'Neill *et al.*, 2001)**.**

Up to 95% of RGII is dimerised in WT plants when sufficient boron is present (Funakawa and Miwa, 2015). Insufficient boron or disruption of the bond results in high levels of RGII monomers (Kobayashi, Matoh and Azuma, 1996). When the boron supply is restricted the majority of boron is seen to accumulate in the cell wall which would suggest this is where it is functional (Ishii and Matsunaga, 1996). The crosslinking of RGII is considered to be the primary role of boron in plants.

1.5.1 RGII in *mur1* and *sfr8*

The lack of fucose within *mur1* and *sfr8* cells causes the L-fucose residues to be replaced with L-galactose (Zablackis *et al.*, 1995). This substitution occurs in side chain A of the cell wall

pectin RGII (Figure 1.3) which causes truncation of the side chain. Side chain A is involved in cross-linking of RGII and the reduction in full length side chains inhibits dimerisation (Pabst *et al.*, 2013; Sechet *et al.*, 2018). While normal levels of RGII are seen in *mur1* mutants only half is dimerised as opposed to at least 90% in WT (O'Neill *et al.*, 2001). This prompted the investigation into the possible link between RGII dimerisation and freezing tolerance.

Studies on *mur1-1* suggest that the altered polysaccharide composition and inhibition of RGII crosslinking causes alterations in cell wall structure such as swelling or thinning of the cell wall. These changes were seen to be reversed when plants were supplemented with boron or L-fucose (Ishii, Matsunaga and Hayashi, 2001; O'Neill *et al.*, 2001; Ryden *et al.*, 2003). WT phenotype and freezing sensitivity were also seen to be restored when supplementation with fucose or boric acid occurred (Panter *et al.*, 2019). The freezing sensitivity of *mur1* and *sfr8* mutants clearly implicates the cell wall in freezing tolerance; this provides some of the first clear evidence that the cell wall plays a role in freezing tolerance.

1.6 Boron in the cell wall

While excess boron can have a toxic effect on plants, deficiency causes anatomical, physiological and biochemical changes within the cell and appears to have a more pronounced effect on cell elongation than cell division (Ishii and Matsunaga, 1996). Boron is an essential trace element for plant growth, as RGII dimerisation is essential to maintain cell wall structure. Insufficient boron leads to a reduction in the quality and quantity of plant produce diminishing crop yields (Miwa *et al.*, 2010).

1.6.1 Boron transport

Boron is transported into the shoots and leaves of the plant via the xylem in the form of boric acid. As boric acid is a small, uncharged molecule it can permeate the lipid membrane with relative ease (Miwa and Fujiwara, 2010). This allows it to be transported through the plant by passive diffusion (Hu and Brown, 1997). However, boron has been seen to accumulate against concentration gradients particularly when under limiting boron conditions (Noguchi *et al.*, 2000). Intrinsic protein channels known as transporters have been found to be required for efficient transport of boron, especially under boron limiting conditions (Miwa and Fujiwara, 2010).

Several important intrinsic proteins are believed to be involved in the active transport of borate throughout the plant. NOD26-like intrinsic proteins (NIPs) are well researched and known to be essential for efficient boron uptake (Figures 1.4 and 1.5) (Takano, Wada, Ludewig, Schaaf, Von Wiré, *et al.*, 2006; Takano *et al.*, 2010). In addition, active transport via proteins

such as BOR1 and BOR2 is essential for boron transport (Takano, Miwa and Fujiwara, 2008; Miwa *et al.*, 2013).

1.7 BOR transporter protein mutants

BOR1 and BOR2 are important efflux boron transporter proteins required for normal transportation of boron throughout the plant. Both high and low boron levels can be damaging to plants therefore plants have evolved to degrade BOR1 transporter proteins when toxic levels of boron are detected preventing transportation through the plant (Takano *et al.*, 2005; Kasai *et al.*, 2010). Mutants have also been designed to target damagingly low levels of available boron; BOR1 over expression mutants. These over-expressers have been shown to require lower boron levels to survive and produce seeds. Under boron limiting conditions they produce higher yields than wild type plants (Miwa, Takano and Fujiwara, 2006).

T-DNA insertion mutants with loss of function of their genes encoding boron transporter proteins BOR1 or BOR2 (*bor2-1* and *bor2-2*) were investigated (Alonso *et al.*, 2003; Rosso *et al.*, 2003). Mutants *requires high boron 1* (*bor1*) and *requires high boron 2* (*bor2*) are known to be defective in their ability to crosslink RGII. They have both been shown to cause reduced root and shoot growth along with leaf expansion although it is believed that these proteins play separate roles in boron transportation (Noguchi *et al.*, 1997; Miwa *et al.*, 2013). The stunted phenotype observed in *bor* mutants may be due to insufficient boron levels limiting RGII dimerisation. Reduced dimerisation is seen in both *bor1* and *bor2* mutants and may cause the stunted phenotype as it is thought to be involved in stabilising the cell wall to allow for cell growth and elongation (Ishii and Matsunaga, 1996).

1.7.1 BOR1

It has been shown that 90% of RGII is in the dimeric form in WT plants whereas in *BOR1* mutants only 30-40% dimerisation was observed under boron limiting conditions (Noguchi *et al.*, 2000, 2003). However, with sufficient boron, dimerisation levels in the mutant were seen to increase dramatically to levels almost as high as those seen in WT plants. This ability to recover RGII dimerisation levels along with the fact that the sugar composition of the RGII side chains was seen to remain unchanged suggests the mutation does not directly affect the ability of RGII to dimerise. This supports the idea that the reduced level of RGII dimerisation seen in *bor1-1* is caused by an inability to transport boron throughout the plant (Noguchi *et al.*, 2003).

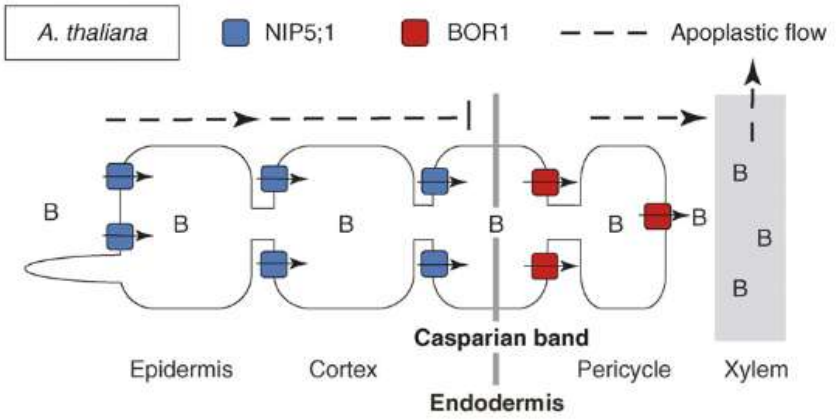

Figure 1.4: Boron transportation through the cell via BOR1. This is the suggested mechanism by which boron is transported by BOR1 against the concentration gradient into the xylem (Takano, Miwa and Fujiwara, 2008).

The low concentration of boron in the stems of *bor1-1* mutants suggest that BOR1 may be involved in transporting boron to the xylem (Figure 1.4) and therefore the shoots of the plant (Noguchi *et al.*, 1997). BOR1 has been shown to localise to the inner plasma membrane domain under boron limiting conditions. This supports the hypothesis that it is involved in transportation of boron to the xylem (Takano *et al.*, 2010).

1.7.2 BOR2

Less research has been carried out BOR2 however, it has also been estabished as an efflux boron transporter localised in the plasma membrane. *BOR2* mutants show a more significant reduction in root elongation under boron limiting conditions but a less severe reduction of shoot elongation when compared to *BOR1* (Takano *et al.*, 2002). Levels of RGII crosslinking were seen to be reduced in the root cells of *BOR2* mutants. Thus, BOR2 is thought to be involved in the transport of borate from the symplast to the apopast (Figure 1.5) to enable RGII dimerisation in root cells and enable elongation (Miwa *et al.*, 2013).

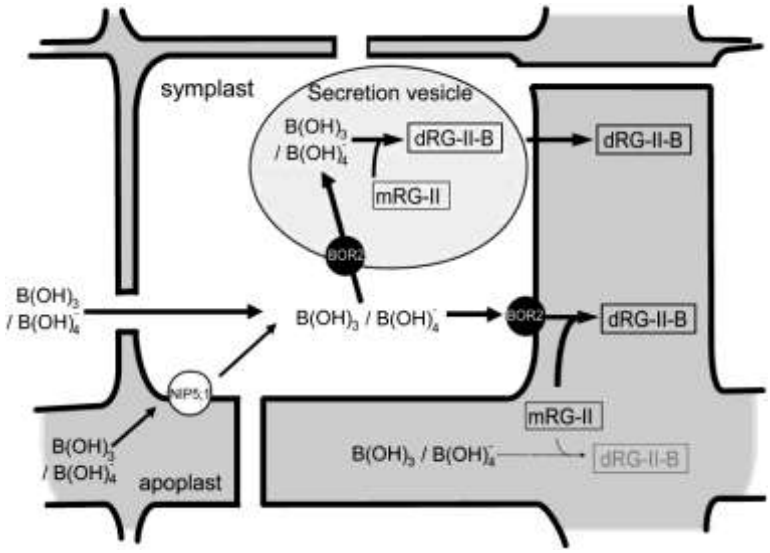

Figure 1.5: Boron transportation through the cell via BOR2. Proposed mechanisms by which BOR2 proteins could transport boron through the cell to allow RGII dimerisation to occur. Currently it remains unclear which pathway is correct, however, dimerised

RGII in the appoplast is known to be the result of BOR2 protein channels (Miwa *et al.*, 2013).

The differences that have been observed between *bor1* and *bor2* support the hypothesis that while both are key proteins for the transportation and distribution of boron they do so via independent pathways (Miwa *et al.*, 2013). In addition, other transporters are thought to play a role in boron transport. NIP5 is an essential transporter required for the uptake of boric acid into the roots (Takano, Wada, Ludewig, Schaaf, Von Wirén, *et al.*, 2006). Once boron has been taken up transport thought the plant may be aided by BOR3, BOR5, BOR6 and BOR7 which have all been identified as potential boron transporter proteins however their exact function is still unknown (Reid, 2014). In addition, it has been suggested that major intrinsic proteins in the plasma membrane, such as PIP1, normally used to transport small neutral molecules may also be used to transport boron under high boron conditions (Dordas, Chrispeels and Brown, 2000).

1.8 Desiccation

One of the major causes of damage and death during freezing is freezing-induced dehydration. This means that plants can respond to drought and freezing in a similar way and that the pathways activated can overlap. As such, given the suggested role of RGII dimerisation in freezing tolerance, drought tolerance was also investigated to determine if RGII dimerisation levels impact the desiccation response.

Water is a finite resource is many regions used for crop growth, therefore drought stress is a major challenge faced by the agricultural industry (Kusaka, Lalusin and Fujimura, 2005; Bernier *et al.*, 2008). Water loss is tightly controlled in plants as drought and desiccation can affect everything from germination to growth and development and can result in major yield losses (Kusaka, Lalusin and Fujimura, 2005; Li *et al.*, 2013). The damage caused by drought stress can vary depending on the growth stage of the plant and the severity and duration of the drought (Ahmad *et al.*, 2009). In addition, some plants show innate resistance to drought that has been utilised in crop development and selective breeding (Bernier *et al.*, 2008; Hussain *et al.*, 2018).

1.9 Stomata

In plants most water loss occurs via the stomata in a process known as transpiration (Zeiger, 1983). Stomata are microscopic pores in the leaf epidermis through which plants control gas exchange. Therefore, stomata have a key role in photosynthesis and regulate water-use efficiency by controlling the exchange of CO_2 and water vapour between the plant and its environment (Cowan and Troughton, 1971; Zeiger, 1983). Plants do this by opening and closing their stomata in response to environmental conditions allowing them to live in a range of environments and survive variation in weather and climate (Araújo, Fernie and Nunes-Nesi,

2011). Stomata are particularly important during drought when plants need to preserve water in order to survive. As such, stomatal function and development are important targets for crop engineering (Bernier *et al.*, 2008; Hussain *et al.*, 2018; Nadeem *et al.*, 2019). This is increasingly important in the face of global warming as the demand for fresh water will only grow as drought conditions increase. Development of crops that either require less water or are more tolerant to drought is becoming ever more important. As such, a more in depth understanding of stomata and the mechanisms that control transpiration may enhance crop development.

1.9.1 Stomatal structure

The stomatal pore is comprised of an opening in the epidermis enclosed by two specialised guard cells. Plants can alter their stomatal aperture to regulate the gas exchange that occurs between the plant and its environment (Cowan and Troughton, 1971; Zeiger, 1983). Stomata open to allow the CO_2 required for photosynthesis to enter the plant; however, this also allows increased water evaporation through the pore. Approximately 90% of the water taken up by plants is lost via transpiration (Schroeder *et al.*, 2001). Therefore, to balance the need for CO_2 against the need to conserve water plants have developed complex mechanisms to regulate gas exchange. In the short-term, plants regulate gas exchange by altering their stomatal aperture. This is done by altering guard cell turgor in response to both biotic and abiotic signals; in particular environmental signals such as light, temperature, humidity, CO_2 and water concentrations (Schroeder *et al.*, 2001; Araújo, Fernie and Nunes-Nesi, 2011).

Guard cell turgor is controlled by ion channel manipulation of osmotic pressure. Environmental signals such as blue light can trigger H^+-ATPase activity (Shimazaki *et al.*, 2007) leading to increased guard cell potassium (K^+) and chlorine (Cl^-) ion uptake. This decreases the cell's osmotic pressure and enables an influx of water into the cells, increasing guard cell turgor and opening the stomatal pore (Shimazaki and Kondo, 1987; Schroeder, 1988). In comparison, the efflux of K^+ ions decreases guard cell turgor and allows stomatal closure (Hosy *et al.*, 2003). Abiotic stress such as drought triggers the complex signalling network induced by abscisic acid (ABA) which ultimately leads to stomatal closure via K^+ efflux (Hetherington, 2001; Li, Assmann and Albert, 2006; Daszkowska-Golec *et al.*, 2013; Pantin *et al.*, 2013). ABA signalling increases cytosolic (Ca^{2+}) levels; decreasing cytosolic pH and causing efflux of K^+ (Schroeder *et al.*, 2001; Li, Assmann and Albert, 2006). This is very important in stomatal movement as increased Ca^{2+} can also actively prevent stomatal opening by inhibition of H^+-ATPase channels (Kinoshita, Nishimura and Shimazaki, 1995). Ca^{2+} signalling can also be triggered by other external stimuli such as CO_2 levels and pathogens which inhibit stomatal opening (Webb *et al.*, 1996; Kuznetsova, Brockhoff and Christensen, 2017).

1.9.2 Stomatal density and distribution

While stomatal aperture can be utilized for short term control of gas exchange, long term alterations can also be made via changes in stomatal density and development as these can have a significant impact on gas exchange (Hepworth *et al.*, 2015; Pirasteh-Anosheh *et al.*, 2016; Bertolino, Caine and Gray, 2019). As such, stomatal patterning and differentiation are carefully controlled and regulated within growth and cell division via a complex network of signals. Environmental cues such as CO_2, light and soil water levels are known to play an important role in this process (Gay and Hurd, 1975; Woodward and Kelly, 1995; Liu *et al.*, 2012). This complex signalling array controls the ratio of stomata to epidermal cells and can be adjusted to environmental conditions as new leaves grow and develop (Woodward and Kelly, 1995). As such, stomatal density can also act as an indicator of environmental conditions and the plants ability to respond to these conditions (Beerling and Chaloner, 1992).

1.9.3 Stomatal variation

Alterations to the complex system that regulates stomata can cause desiccation rates to be dramatically altered. Decreased stomatal density can improve water use efficiency (Caine *et al.*, 2019). Similarly, alterations to epidermal patterning factors can impact stomatal density and drought tolerance (Hepworth *et al.*, 2015). In addition, defective responses to external or internal stimuli or interruptions to signalling pathways such as ABA or salicylic acid can alter water loss both positively and negatively (Saez *et al.*, 2006; Khokon *et al.*, 2017). Structural alterations can also impact transpiration: reduced stomatal aperture can improve drought tolerance (Miura *et al.*, 2013). However, structural alterations may also be detrimental to plants. For example, the lack of cuticular ledge seen in *scord6* mutants may increases pathogen infection rates (Zhang *et al.*, 2011).

1.10 Aims

Evidence to date strongly suggests that the cell wall has a role in protecting plants against freezing damage. Recent studies have shown that cell wall mutants with defective fucose synthesis have reduced freezing tolerance and the lack of RGII dimerisation these mutants display has been implicated in this freezing sensitivity. Further investigation is required to establish if the lack of RGII dimerisation or fucose synthesis is responsible for the increased freezing sensitivity observed. In addition, freezing sensitivity is often seen in conjunction with drought sensitivity, indicating alteration to the cell wall and/or RGII dimerisation may also have an impact on drought tolerance.

Mutants with altered RGII dimerisation were used to investigate the effect that disrupting RGII dimerisation had in both drought and freezing. It was observed that RGII dimerisation is required for freezing tolerance, however, it is not involved in the acclimation process plant

utilize to prepare for freezing. In addition, results indicate that RGII dimerisation may be involved in stomatal function and development which may in turn affect drought tolerance.

Developing a better understanding of the cell wall and in particular RGII dimerisation has highlighted the importance of RGII in freezing and drought tolerance. This improves our understanding of the cell wall and the important role it plays in protecting against environmental stresses. In addition, these findings highlight RGII dimerisation as a potential area of interest for the development of drought or frost resistant crops.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant materials and growth conditions

2.1.1 Seed material

All *Arabidopsis thaliana* plants used were in the Columbia (Col-0) background. *sfr8* and *mur1-1* lab seed stocks were used (Reiter, Chapple and Somerville, 1993; Thorlby *et al.*, 1999). The T-DNA insertion mutants: *bor1-3* (SALK_037312), *bor2-1* (SALK_056473), *bor2-2* (GAB1527H04) and *bor1-3/bor2-1* were obtained from Hokkadio University (Miwa et al., 2013).

Gene name	Full name	AGI number	Alleles
MUR1 or SFR8	MURUS1 or SENSITIVE TO FREEZING 8	AT3G51160	*mur1-1* *sfr8*
BOR1	REQUIRES HIGH BORON 1	AT2G47160	*bor1-3*
BOR2	REQUIRES HIGH BORON 2	AT3G62270	*bor2-1* *bor2-2*

Table 2.1: Mutants under investigation. Full names, alleles and AGI numbers of mutants used throughout experiments.

2.1.2 Soil growth conditions

Seeds were sterilized by shaking in 1 ml of 70% ethanol for 5 min then allowed to dry on sterile filter paper in a laminar flow hood. Seeds were then transferred to 9-cm circular petri dishes containing sterile media. Unless otherwise stated the media used was comprised of 0.8% plant tissue culture grade agar (Sigma Aldrich, Poole, UL) and supplemented with ½ x Murashige and Skoog (MS) media (Murashige and Skoog, 1962). The pH of the media was adjusted to 5.8 before autoclaving at 120°C for 20 min to sterilise. Seeds were stratified in the dark at 4°C for 48-96 h to promote uniform germination and then transferred to a Percival CU-36L5D growth chamber (CLF Plant Climatics, Wertingen, Germany). Growth chamber conditions were set to 16 h light, 8 h dark at 20°C with light intensity of 150 µmol/m^2/s.

To grow mature plants, seedlings were transferred to Jiffy pellets with a 44 mm diameter (LBS Horticulture, UK) at 10-12 days old. Trays were covered with cling film for 2-3 days to allow the plants to acclimatise to the change in humidity. Plants were grown to 5 weeks old and watered every 3-4 days. Unless otherwise stated short day conditions of 12 h light, 12 h dark at 20°C

with a maximum of 70% humidity were used. Plants grown under long day conditions were subjected to 16 h light, 8 h dark at 20°C

2.1.3 Hydroponic growth conditions

A method outlined by Conn (Conn *et al.*, 2013) was used to determine the ease of supplementation via hydroponic growth. All equipment used was autoclaved to ensure a sterile growth environment and liquid media was covered to prevent exposure to light and discourage algal growth. Holes were punched into black centrifuge tube lids which were then filled with agar containing ½ or ¼ x MS with 2-3 seeds placed in each hole to ensure germination. Seeds were stratified for 3-4 days then lids were placed in an empty tip box filled with liquid media containing either ½ or ¼ x MS (Figure 2.1A). They were covered in cling film to ensure humidity and transferred to a Percival growth chamber (16 h light, 8 h dark at 20°C). As seeds germinated they were thinned to ensure only one seedling remained per lid. At approximately 21 days seedlings were transferred to larger boxes containing 50-ml centrifuge tubes with the bottoms cut off to enable the roots of each seedling to remain separate (Figure 2.1B). Plants were then moved to the growth room and exposed to short day conditions of 12 h light and 12 h dark at 20°C for the remainder of their growth. Liquid medium was replaced every week.

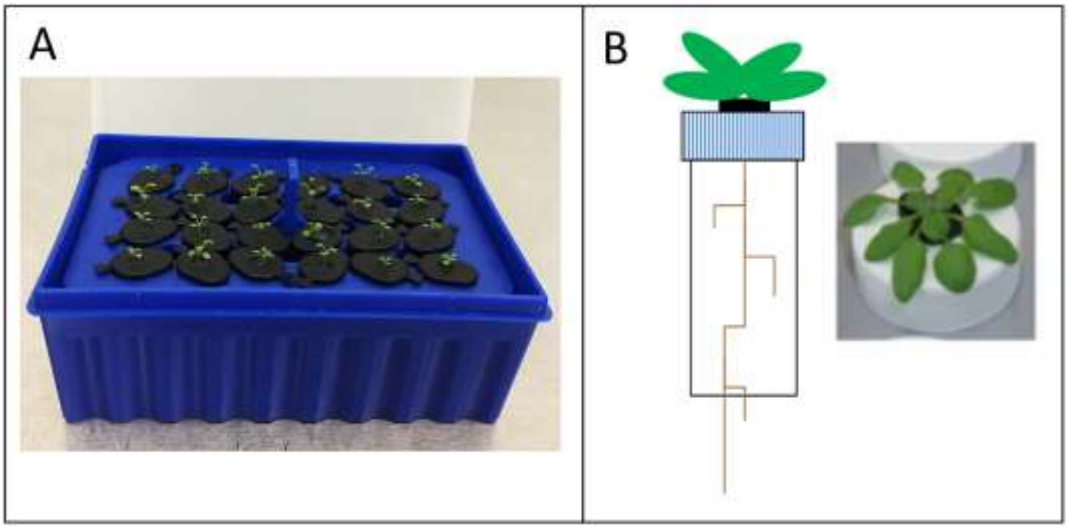

Figure 2.1: Set up of hydroponic growth conditions. A) 10-day-old seedlings. **B)** schematic diagram and aerial image of 5-week-old hydroponically grown plants (Adapted from (Conn *et al.*, 2013)).

2.1.4 Bleach sterilisation

This sterilisation procedure ensures minimal contamination. Seeds were placed in a 50-ml centrifuge tube and shaken in 70% ethanol for 5 min. Ethanol was removed and replaced with a solution of 10% bleach and 0.25% SDS, diluted in dH_2O, and shaken gently for 10 min. Seeds were then washed in sterile H_2O 6 times and then suspended in fresh sterile H_2O. Seeds were

then pipetted directly onto plates and stratified, germinated and grown as previously described.

2.2 Supplementation

2.2.1 Boric acid (BA) supplementation

Seeds were grown on ½ x MS agar supplemented with 1 mM boric acid (BA) for 10-12 days then transferred to peat plugs and watered with 20 mg/L BA.

2.2.2 Fucose supplementation

Seeds were grown on ½ x MS as before and transferred to peat plugs. Once transferred plants were watered normally and sprayed with 60 ml of 10 mM fucose once a week.

2.2.3 Cold acclimation

5-week-old plants were transferred to a MLR-351 environmental test chamber (Sanyo, Osaka, Japan) and exposed to acclimating conditions of 5°C with a light cycle of 10 h light, 14 h dark for 2 weeks.

2.3 Assessment of freezing damage

2.3.1 Plate freezing - qualitative visual freezing assay

Seeds were grown on ½ x MS media in a Percival, as previously described, for 2 weeks. The seedlings were then transferred to a dark freezing chamber where the lids were removed to allow the cold air to interact with the plants. The temperature was dropped one degree every hour starting at 0°C until the test temperatures were reached and plates were removed. The temperatures used for non-acclimated plants were -5°C, -6°C and -7°C. Plants were allowed to recover in the dark at 5°C overnight in an environmental growth chamber to avoid temperature shock and then returned to the Percival growth chamber to observe the effect of freezing over the course of the following week.

2.3.2 Cold Acclimated plants

Acclimated plants were subjected to a minimum of 3 days of acclimating conditions, 5°C in an environmental growth chamber as previously described, prior to freezing. Test temperatures used for these plants were -8°C, -9°C and -10°C.

2.3.3 2F-fucose treatment

Peracetylated 2-fluro 2-deoxy-L-fucose (2F-fucose - Merck Millipore, Darmstadt, Germany) was dissolved in DMSO to 10 mM. Seeds were grown on ½ x MS with 2 % sucrose and supplemented with either 2.5 µM or 10 µM 2F-fucose inhibitor using DMSO as a control. Due to its temperature sensitive nature 2F-fucose was added after the media had been autoclaved

and allowed to cool. Seeds were stratified, germinated and grown as previously described prior to freezing.

2.3.4 Revision to the plate freezing assay

This protocol was revised, and 2% sucrose was added to the growth media to help maintain the integrity of the media post freezing and prevent formation of a water layer. The addition of sucrose caused increased contamination post freezing that interfered with gathering clear results. The level of contamination was reduced by letting the plants remain at 5°C in the environmental growth chamber while observing the effects of freezing. The test temperature ranges were also narrowed to focus on the four most interesting temperatures: -4°C and -5°C for non-acclimated plants and -9°C and -10°C for acclimated plants.

2.3.5 Electrolyte leakage (EL) assay – quantitative freezing assay

Experiments were carried out on 5-week-old plants which were deemed to be the appropriate age for this assay as they have well-developed rosette leaves but are not yet flowering (Hemsley *et al.*, 2014). For acclimation experiments plants were grown for an additional 2 weeks at acclimating temperatures so were 7-weeks-old. Despite being 2 weeks older acclimated plants remain a similar size to non-acclimated 5-week-old plants as growth is limited in acclimating conditions. This ensured acclimated and non-acclimated plants were similar in size allowing the two to be compared more easily.

The size of the freezing bath used limited the experiment to 72 test tubes; this allows 4 genotypes to be investigated at 3 temperatures with each measurement replicated 6 times. Each test tube contained 3 leaf discs excised from 3 leaves of comparable size on an individual plant. Test tubes were stored on ice until all the samples were collected. Leaf discs were then rinsed with dH_2O and all remaining water was gently blotted off with tissue. Test tubes were place in a randomised order into a freezing bath at -2°C and allowed to equilibrate for 1 h. An ice chip of dH_2O was then added to each test tube to initiate freezing and the test tubes were left for a further 2 h at -2°C. The temperature was then lowered to the first test temperature and after 30 min the first set of tubes (6 tubes for each genotype) were removed and placed on ice. This was then repeated for the next two temperatures with a set of tubes being removed after 30 min spent at each temperature. The samples were then thawed at 5°C overnight in a cold chamber. When investigating non-acclimated plants temperatures of -3°C, -5.5°C and -8°C were used. When investigating a combination of acclimated and non-acclimated plants temperatures of -5°C, -7°C and -9°C were used.

The next day 5 ml dH_2O was added to each tube and tubes were shaken gently for 3 h at room temperature. The liquid was decanted into respective tubes and conductivity was measured via a hand held conductivity meter (Mettler Toledo, Columbus, OH, US) Tubes containing the

leaf discs were frozen at -80°C for a minimum of 1 h to fully destroy leaf tissue causing all ions to be released from the tissue. The liquid was then returned to the tubes containing their respective leaves and shaken again at room temperature for 3 h. Leaves were removed and conductivity was measured again, this allowed quantification of the level of electrolyte loss caused by the initial freezing event to be expressed as a percentage of the total electrolyte content of the tissue tested. This was calculated by expressing the first reading as a percentage of the second.

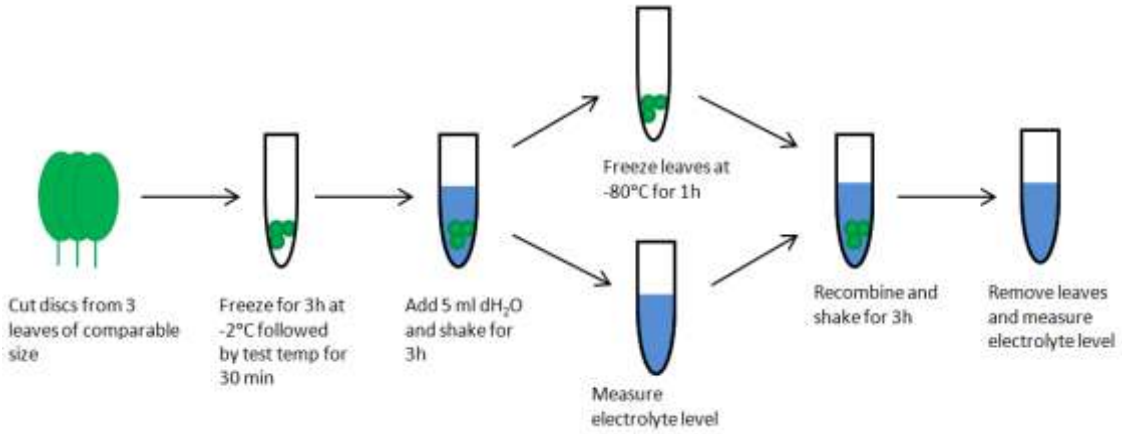

Figure 2.2: Electrolyte leakage (EL) protocol. Schematic outline highlighting the key steps of the EL protocol.

This protocol was altered for mutants with reduced size; as their leaves were too small to excise leaf discs whole leaves were used instead. Due to the limited size of the freezing bath when investigating 5 genotypes at once 2 temperatures (-4°C and -6°C) were used to enable each measurement to be replicated 6 times.

2.4 Assessment of RGII dimerisation

2.4.1 Alcohol insoluble residue (AIR) prep

Tissue was harvested from three 5-week-old plants of each genotype or treatment. The tissue was ground into a fine powder in liquid nitrogen using a pestle and mortar. Fifty mg (+/- 1 mg) of powder was placed in a 1.5-ml centrifuge tube with 1 ml 100% ethanol and shaken at 250 rpm at room temperature for 4 h. Samples were centrifuged at 16000 *g* for 3 min and the supernatant was removed. One ml fresh 100% ethanol was added and the samples were shaken as before at 37°C overnight (16 h). Samples were centrifuged as before and supernatant removed before samples were dried on a heat block at 37°C to allow all the ethanol to evaporate (approx 6 h). One mg dried tissue was transferred to a clean 1.5-ml centrifuge tube and centrifuged to collect the powder at the bottom. Samples were incubated in 350 µl Na_2CO_3 at 4°C overnight (16 h) shaking gently. Samples were centrifuged as before and supernatant removed. Tissue was neutralised with excess acetic acid (100 µl) and washed with 500 µl 75% ethanol, followed by 3/4 washes with dH_2O.

2.4.2 Digestion with endo-polygalacturonase (EPG)

100 µl of 10 U/ml endo-polygalacturonase (EPG - Merck, Darmstadt, Germany) in pyridine/acetic acid/0.5% chlorobutanol (1:1:98, pH 4.7) was added and tubes were shaken at room temp overnight (16 h).

2.4.3 Gel electrophoresis and silver staining

Gel electrophoresis and silver staining was carried out as described by Chormova (Chormova, Messenger and Fry, 2014). A 7.5-ml 26.4% polyacrylamide gel was made by mixing: 1.251 ml 1.5 M Tris (pH8.8), 6.246 ml 40% acrylamide/bis acrylamide (29:1), 70.05 µl 10% APS, 5.85 µl TEMED. The gel was pipetted into the gel casting apparatus and allowed to solidify before being placed in electrode buffer (50 mM Tris/38 mM Glycine). Samples were centrifuged at 16000 *g* for 3 min and 10 µl of the supernatant was mixed with 2 µl loading buffer (0.6 M Tris HCl/0.25% w/v bromophenol blue/50% glycerol, pH8.8). Samples were loaded onto the gel and run at 200 V, 80 mA and 50 W at room temperature for 75-90 min. The gel was then removed from the glass plates, placed in a tray and fixed in ethanol/acetic acid/H_2O (4:1:5) by shaking gently for 30 min. It was then washed with H_2O for 1 min, three times before being treated with 400 µM sodium thiosulphate for 1 min. This was followed by a further three washes with H_2O for 1 min. The gel was stained by shaking gently in a solution of 6 mM $AgNO_3$/10 mM formaldehyde for 20 min. The stain was then removed by washing twice with H_2O for 20 s and colour was developed by adding a solution of 0.28 M Na_2CO_3/8 µM sodium thiosulphate/64 mM formaldehyde. This was shaken gently until colour started to develop, the solution was replaced with fresh solution to enhance colour development and continued to be shaken gently until adequate contrast was achieved. Colour development was then stopped by adding 0.33 M Tris/2% acetic acid and the gels were scanned or photographed.

2.5 Assessment of leaf water loss

2.5.1 Leaf drying assay

Mature 5-week-old plants were used in this experiment. The day before the experiment was carried out plants were watered thoroughly and covered with a plastic bag to ensure a 100% humid environment. Seven plants were used per genotype or treatment and 1 leaf was removed from each plant, blotted dry and weighed immediately. Leaves were then placed abaxial side up in a weighing boat. Leaves were kept on a bench in the lab at room temperature (approximately 22°C, 50% humidity) and weighed every hour for 8 hours to investigate the rate at which water was lost. Additional measurements were taken after 48 h and 7 days. At 7 days this measurement is taken to be the dry weight of the leaves; this allows the percentage water weight of the leaf to be calculated. Measurements were expressed as a percentage of the original weight.

2.5.2 Thermal imaging

5-week-old plants were watered thoroughly and covered with a plastic bag the night before the experiment to ensure an environment of 100% humidity. Plants were placed in an alternating fashion (see Figure 3.14) onto a matte black surface and allowed to acclimate to the change in humidity for 40 min; four plants from each genotype were used. A FLIR T1030sc infrared camera (FLIR systems, USA) was placed at a distance of approximately 1 m and plants were photographed at 30 min intervals for 4 days in constant light. Emissivity was set at 0.95, ambient temperature at 23°C and humidity at 50%.

2.6 Assessment of Stomatal morphology and distribution

2.6.1 EM sample preparation

5-week-old plants were cut into small sections, approximately 2 mm by 6 mm, and fixed in Karnovsky's fixative (2% PFA, 2.5% glutaraldehyde, 0.1 M phosphate buffer, pH 7.5) at room temp for 1.5 h, shaking gently. Samples were washed with 0.1 M cocodylate buffer twice for 2 min and stained with 1% buffered osmium tetroxide for 1.5-2 h at room temp. An ethanol series was used to dehydrate the samples; 25%, 50%, 75% and 95% three times for 5 min each. This was followed by three times 100% ethanol for 10 min.

Samples were then split and processed for either scanning electron microscopy or embedded in resin for cross sectioning.

2.6.2 Scanning electron microscopy (SEM)

Samples were dried in a CPD030 critical point dryer (BAL-TEC, Pfäffikon, Switzerland) using liquid carbon dioxide as the transitional fluid. Samples were then mounted on silicon chips (Agar Scientific, Essex, UK) and coated with platinum (~10 nm thickness) in a 328 sputter coating system (Cressington, Watford, UK).

Samples were examined using a S-5200 scanning electron microscope (Hitachi, Tokyo, Japan) at 15.0 kV accelerating voltage. Approximately 40 images of individual stomata from 4 plants per genotype or treatment were taken at a magnification of x3.5k.

2.6.3 Embedding leaves in resin

Samples were infiltrated with resin starting with a 50:50 mix of 100% alcohol and propylene oxide three times for 10 min followed by 100% propylene oxide three times for 10 min. Samples were then incubated in 50:50 100% propylene oxide/araldite overnight (approx 16 h) at room temp with the lids off, followed by two washes in 100% resin for 1 h. Finally, samples were embedded in coffin moulds, covered with fresh 100% araldite and polymerized at 60°C for 24 h.

Five hundred-nm cross-sections were cut from the resin blocks using glass knives on a UC6 Ultramicrotome (Leica, Wetzlar, Germany). Samples were then placed on a glass slide and stained with 1% toluidine blue and imaged via light microscope. 10-15 images were taken per genotype or treatment at a magnification of x100.

2.6.4 Transmission electron microscopy (TEM)

Resin samples were also used for TEM imaging. Due to time restraints imaging was carried out by Durham University electron microscopy department on a H76000 transmission electron microscope (Hitachi, Tokyo, Japan).

2.6.5 Stomatal density

Twelve SEM images with x400 magnification were taken per genotype showing an area of approximately 330 µm by 230 µm. The number of stomata in each image were counted and an average was taken. Samples were provided from 4 plants per genotype or treatment

2.7 Molecular biology techniques

2.7.1 gDNA extraction

A modified version of the method outlined by Edwards (Edwards, Johnstone and Thompson, 1991) was used to extract gDNA. Individual seedlings were placed in 1.5-ml centrifuge tubes and frozen in liquid nitrogen. Samples were ground with an electronic micropestle and then homogenised in 400 µl of Edward's extraction buffer (200 mM tris-HCL, pH 7.5/250 mM NaCl/25 mM EDTA, pH 8/ 0.5% SDS). Samples were vortexed to ensure thorough mixing and centrifuged at 16000 *g* for 1 min. 300 µl of supernatant was transferred to a fresh 1.5-ml centrifuge tube and mixed with 300 µl isopropanol before being incubated at room temperature for 2 min. Samples were centrifuged at 16000 *g* for 5 min and the supernatant was removed. Samples were centrifuged again, at the same speed, for 1 min and all remaining supernatant was removed before the samples were dried in a Concentrator 5301 vacuum desiccator (Eppendorf, Hamburg, Germany) for 5 min. 50 µl of TE buffer (10 mM Tris, pH 8, 1 mM EDTA) was added to resuspend the DNA and the samples were incubated for 72 h at 5°C to allow the pellet to re-suspend and the DNA to dissolve into the solution.

2.7.2 Polymerase chain reaction (PCR) of *MUR1* gene for genotyping purposes

A region of the *MUR1* gene was amplified from gDNA via PCR using DreamTaq DNA polymerase. A 50-µl reaction was made up in a 500-µl microcentrifuge tube; 5 µl of 10 x DreamTaq green buffer, 1 µl 10 mM dNTPs, 0.25 µl forward primer (50 µM), 0.25 µl reverse primer (50 µM), 0.25 µl DreamTaq polymerase, 1 µl DNA and 42.25 µl nuclease-free H_2O. Samples were placed in a 96-well PCR Express thermocycler (Thermo Hybaid, Thermo Scientific,

Wilmington, DE, US) and run on the following programme; 95°C, 2 min x 1; (95°C, 30 s; Ta, 30 s; 72°C, 1 min) x 35; 72°C, 5 min x 1. PCR products (approx 320 bp) were analysed using gel electrophoresis.

2.7.3 Gel electrophoresis

Agarose gel electrophoresis was carried out to separate DNA by size. Molecular biology grade agarose was melted in 0.5 x TBE buffer (1.1M Tris, 900 mM borate, 25 mM EDTA, pH 8) to give a 0.8-% gel and 2 µl MIDORI^{GREEN} was added per 50 ml agarose. A running buffer of 0.5 x TBE (Tris base, boric acid, EDTA, pH 8.3) was used and 10 µl PCR product was loaded into each well along with 5 µl of 1Kb Hyperladder 1 (Bioline, Nottingham, UK). The gel was run at 35 mA for approximately 1 h and imaged under UV.

2.7.4 DNA clean up

DNA products were purified for sequencing using E.N.Z.A cycle pure kit (Omega bio-tek, Norcross, GA, US). Samples were placed in clean 1.5-ml centrifuge tubes and 5 times the volume of CP buffer was added before vortexing thoroughly and centrifuging to collect the sample. Samples were transferred to HiBind DNA minicolumns and centrifuged at 16000 *g* for 1 min and the filtrate was disposed of. 700 µl DNA wash buffer, diluted with 100% ethanol, was added to the column before centrifuging as before for 1 min and disposing of filtrate. A further 700 µl DNA wash buffer was added and this step was repeated. The empty column was centrifuged as before for 3 min to dry the column and then transferred to a clean 1.5-ml centrifuge tube. 30 µl elution buffer was added to the centre of the column and incubated at room temp for 2 min and then centrifuged as before for 2 min. Samples were concentrated via vacuum concentrator and DNA concentration was measured via ND-1000 Spectrophotometer (NanoDrop, Thermo Scentific, Wilmington, DE, US).

2.7.5 Genotyping *sfr8* mutants

The varied phenotypes observed in *sfr8* can make it difficult to visually confirm the plants genotype. To ensure the reliability of the seed lines plants were genotyped when bulking seed stocks. This was confirmed via sequencing of part of the *MUR1* gene as shown by Panter et al, (2019). *sfr8* mutant plants display a single base change from G to A at nucleotide 629 of the gene. 3 plants thought to be WT and 3 thought to be *sfr8* were tested. Nucleotide and amino acid sequences can be seen in Figure 1.1.

2.7.6 Sequencing

Once the DNA was cleaned up the PCR product was sequenced using the forward primer used for amplification (at a concentrate of 3.2 µM) by the DBS Genomics (Department of Biosciences, Durham University).

Sequences were examined using Chromas (https://technelysium.com.au/wp/chromas/).

2.8 Statistics

2.8.1 EL statistics

R studio (https://www.rstudio.com/) software was used for electrolyte leakage statistical analysis. Percentage electrolyte leakage values from 3 biological replicate experiments were used unless otherwise stated. Percentage values were arcsine-transformed to follow a normal distribution and a linear mixed effects model (Kuznetsova, Brockhoff and Christensen, 2017) was used. Genotype, size or supplementation were specified as fixed terms while the experiment was specified as a random effect. A one-way ANOVA was used to determine the effect the varying factor on the level of electrolyte leakage. Significant difference in leakage at each temperature was assessed by least-squares means comparison (Lenth, 2016).

CHAPTER 3

RESULTS

3.1 Freezing sensitivity and RGII dimerisation

3.1.1 Freezing sensitivity of *sfr8* mutants

The *sfr8* mutant was identified in a screen for cold-acclimation mutants (Warren *et al.*, 1996). Further research has quantified *sfr8* freezing sensitivity, however, these experiments were all carried out on cold-acclimated plants (Panter, 2019). Cold acclimation occurs when plants are exposed to low, non-freezing temperatures for a period of time - typically 2 weeks for mature plants and 3-5 days for seedlings. Acclimation can result in biochemical, molecular and physiological changes within the plant that can enable it to be more resilient to freezing events. As these experiments were carried out on acclimated plants it is unclear whether the freezing sensitivity observed in *sfr8* mutants is caused by an inability to acclimate or an inherent freezing sensitivity. To determine whether *sfr8* is a freezing-sensitive mutant or a cold acclimation mutant, freezing tolerance of both acclimated and non-acclimated *sfr8* and WT plants were compared.

3.1.2 Qualitative assessment of the freezing damage in acclimated vs. non-acclimated *sfr8* plants

Plate freezing assays were carried out to qualitatively assess freezing sensitivity. The ability of both acclimated and non-acclimated 14-day-old seedlings to survive and recover from a freezing event was investigated. Lab stocks of *sfr8* were used but due to the phenotypic similarities between *sfr8* and WT genotyping was carried out to ensure the correct lines were in use. Each plate was sewn with both WT and *sfr8* seedlings to allow for direct comparison and plates were either frozen directly after two weeks of growth at 20°C or allowed to acclimate for 3-4 days prior to freezing. Plants were then allowed to recover at 20°C and the level of recovery was assessed. The survival rate was used as an indicator of the degree of damage caused by freezing. Initial experiments were carried out at -5°C, -6°C and -7°C for non-acclimated plants and -8°C, -9°C and -10°C for acclimated plants as acclimated plants have higher freezing tolerance.

Acclimated *sfr8* seedlings were less able to recover than acclimated WT seedlings after freezing at all temperatures observed indicating that, when acclimated, *sfr8* seedlings are more sensitive to freezing than WT (Figure 3.1A). This result correlates with qualitative results observed in previous freezing-sensitivity experiments (Panter *et al.*, 2019) indicating that the assay is a reliable measure of freezing sensitivity. Reduced recovery after freezing was also

seen in non-acclimated *sfr8* seedlings frozen at -4°C (Figure 3.1B). This indicates that non-acclimated *sfr8* mutants are also more sensitive to freezing than their WT control. This would suggest that the freezing sensitivity observed in *sfr8* mutants is intrinsic and not a result of an inability to acclimate. This is corroborated by the fact non-acclimated *sfr8* seedlings appear dead at temperatures as high as -4°C whereas once acclimated they are able to survive temperatures as low as -9°C. These results indicate that *sfr8* mutants are inherently more freezing-sensitive than WT but are capable of undergoing successful acclimation up to a point.

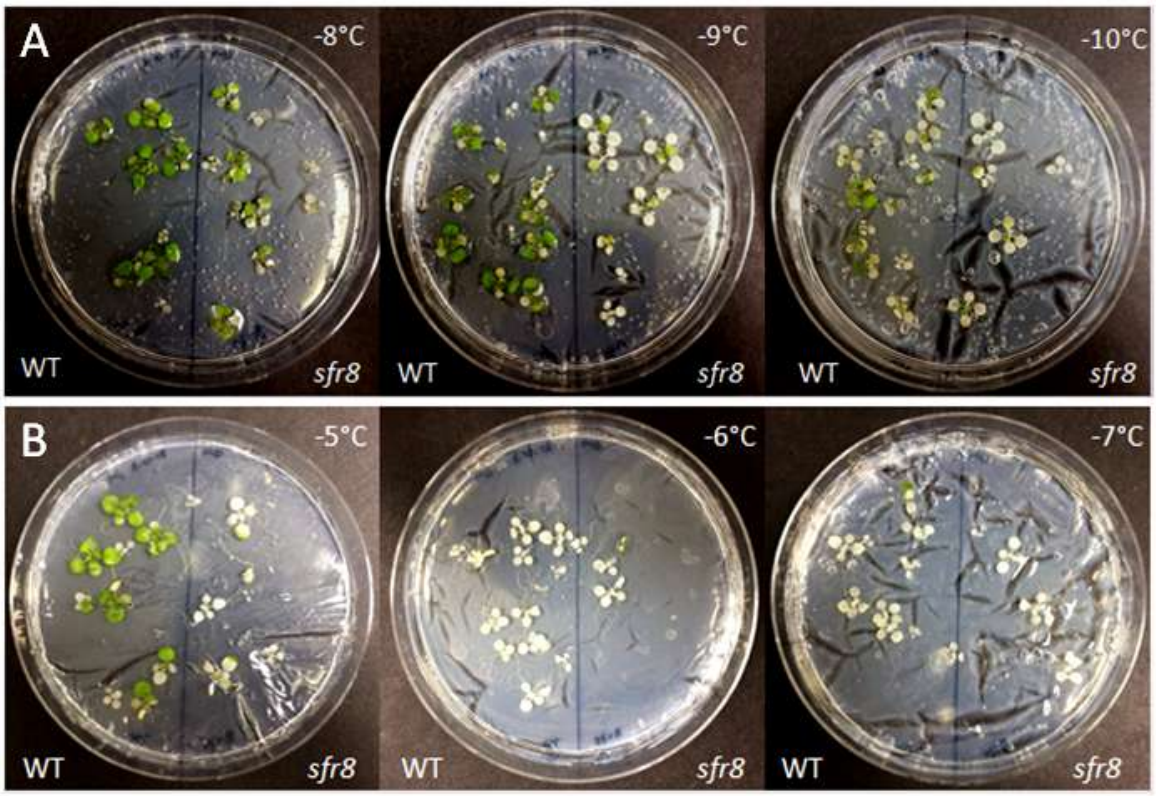

Figure 3.1: Plate freezing assay to assess freezing damage on acclimated and non-acclimated WT and *sfr8* seedlings. Both Col-0 (WT) and *sfr8* seedlings were grown on ½ x MS. **A)** Seedlings were grown for 14 days followed by a period of acclimation at 5°C for 3 days. Plates were then frozen at -8°C, -9°C or -10°C and plates were then allowed to recover at 20°C and assessed after 3-5 days. **B)** Seedlings were grown for 14 days and frozen, without acclimation, at -5°C, -6°C or -7°C. Plates were then allowed to recover at 20°C and assessed after 3-5 days.

When plates were thawed post-freezing the media split into a thin agar layer and a watery layer. Therefore, the results observed might have been caused by *sfr8* seedlings becoming submerged due to their shortened petioles leading to anoxia and an increase in damage or death rates. To verify whether the damage observed in the seedlings was a result of the freezing or anoxia the experiments were repeated on plates containing 2% sucrose. The addition of sucrose has been shown to allow the agar to retain its structure post freezing (Huang *et al.*, 2017). In this experiment the temperature range used was adjusted to -9°C and -

10°C for acclimated plants and -4°C and -5°C for non-acclimated plants since this was the range in which a difference in recovery was found. The results observed replicated those seen in the previous experiment: *sfr8* seedlings were seen to be more sensitive to freezing when both acclimated and non-acclimated and *sfr8* is better able to withstand freezing after a period of acclimation. This confirmed that the differences in recovery observed in the first experiment were due to increased freezing sensitivity and not submersion (Figure 3.2). Since the addition of sucrose to the plates lead to increased yeast colonisation, these plates were allowed to recover at 5°C rather than 20°C. While this prevented contamination with yeast, the stress of a lower recovery temperature led to a build up of anthocyanin in the leaves as seen in Figure 3.2.

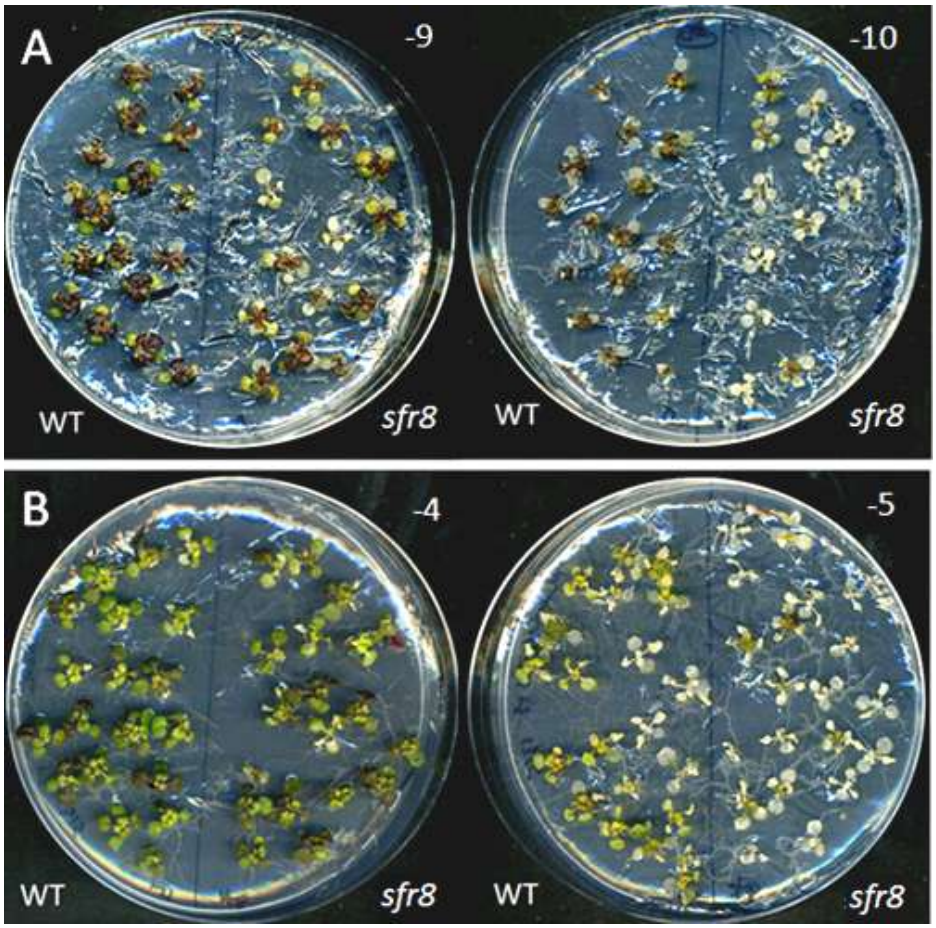

Figure 3.2: Plate freezing assay to assess freezing damage on acclimated and non-acclimated WT and *sfr8* seedlings on agar with additional sucrose. Seedlings were grown on ½ x MS supplemented with 2% sucrose. Each plate contained both *sfr8* and Col-0 (WT) and was replicated 4 times, a representative image is shown. **A)** Seedlings were grown for 14 days followed by a period of cold acclimation for 3 days. Plates were then frozen at -9°C or -10°C and plates were then allowed to recover at 20°C and assessed after 3-5 days. **B)** Seedlings were grown for 14 days and frozen, without acclimation, at -4°C or -5°C. Plates were then allowed to recover at 5°C and assessed after 3-5 days.

3.1.3 Quantitative analysis of freezing damage in acclimated vs. non-acclimated *sfr8* plants

Electrolyte leakage (EL) assays were used to quantify the increased freezing sensitivity of *sfr8* seedlings observed in the plate freezing assays. Experiments were carried out on 5-week-old plants grown at 20°C or on plants with an additional 2 week cold-acclimation period at 5°C. Figure 3.3A shows the results of EL using both acclimated and non-acclimated plants. However, the wide temperature range needed to encompass both acclimated and non-acclimated plants caused some difficulty in getting results from both sets of plants in the same experiment. Acclimated plants show very little damage at -5°C whereas non-acclimated plants show almost 100% leakage at -8°C. Therefore, triplicate data from both acclimated (Figure 3.3B) and non-acclimated (Figure 3.3C) plants are shown separately. These experiments each had 6 biological replicates per genotype and per temperature with 3 leaf discs used per biological replicate. This gives a qualitative measure of the damage inflicted on cells by freezing leaves at various temperatures both with and without a period of cold-acclimation ((Gilmour, Hajela and Thomashow, 1988). The portion of total cellular electrolytes released from leaf discs frozen at 3 temperatures and then thawed can be taken as a measure of damage. This allows the freezing tolerance of the plant to be measured. To assess the significance of these results a one-way ANOVA was carried out followed by a least squared means (LSM) comparison.

When frozen, both WT and *sfr8* leaves showed significantly higher levels of leakage, and therefore damage, when non-acclimated than when acclimated (P <0.001) (Figure 3.3A). This shows that *sfr8* is also able to undergo cold-acclimation and can successfully increase its freezing tolerance. Acclimated *sfr8* leaves also display significantly higher levels of damage (P <0.001 at -7°C and -9°C) than acclimated WT leaves. Non-acclimated *sfr8* also show significantly higher levels of damage than WT (P <0.001 at -5°C and -7°C and P >0.05 at -9°C). This has enabled the results seen in Figures 3.1 and 3.2 to be quantified. Similar results were seen in separate acclimated (Figure 3.3B) and non-acclimated (Figure 3.3C) triplicate data: *sfr8* showed significantly higher levels of damage than WT in both acclimated (P <0.001 at -5°C and -7°C, P <0.01 at -9°C) and non-acclimated (P <0.001 at -5°C and -7°C, P >0.05 at -9°C) leaves. This indicates that *sfr8* plants are inherently more freezing-sensitive than WT. This concurs with the qualitative results seen in (Figures 3.1 and 3.2).

The difference between *sfr8* and WT appears to diminish as the temperature decreases. However, this is likely to be caused by leaves reaching a maximum level of leakage as percentage leakage never reaches 100% even when leaves are completely destroyed.

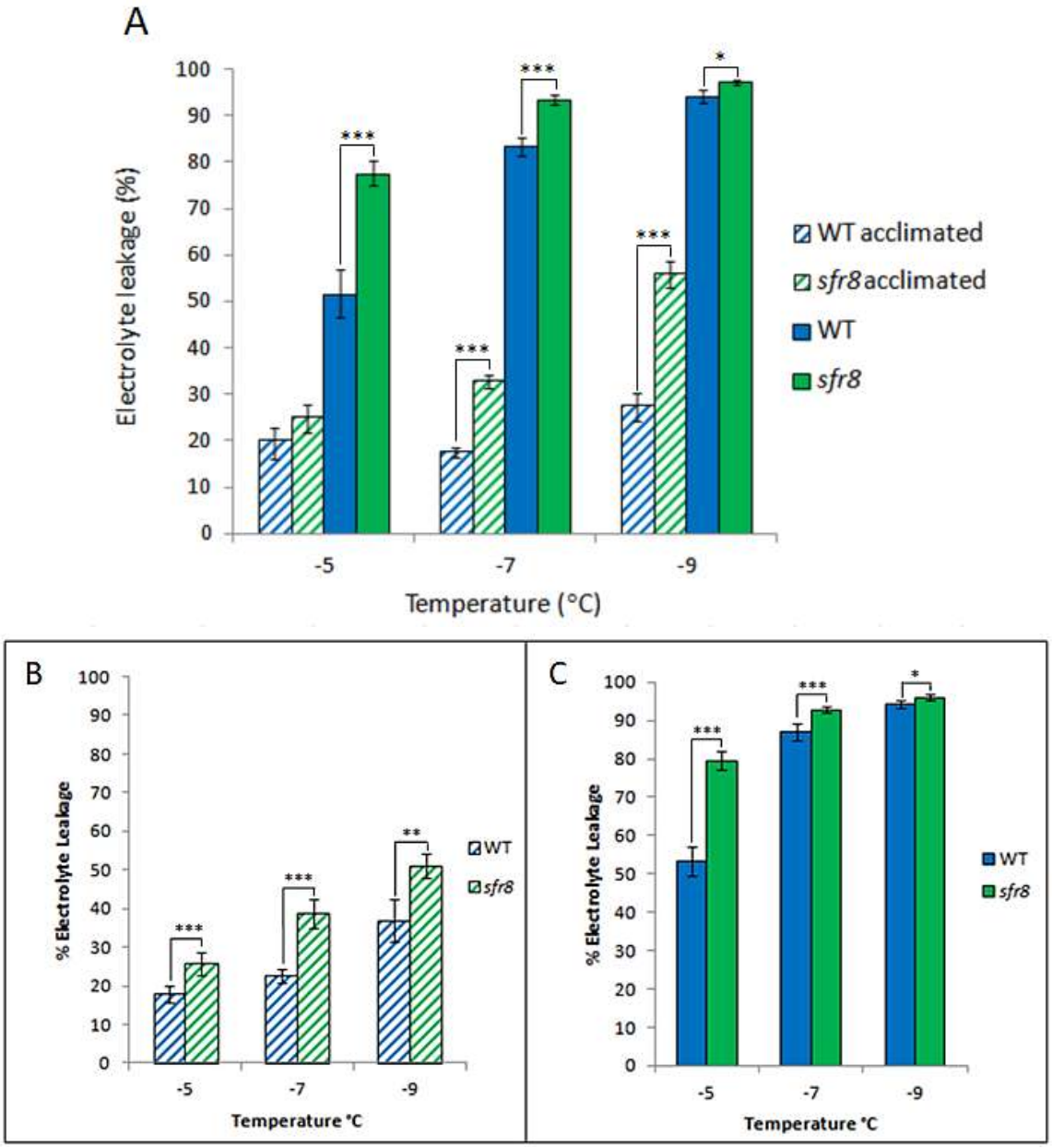

Figure 3.3: Electrolyte leakage from leaf discs of WT and *sfr8* mutant plants. Values indicate percentage of electrolytes lost from Col-0 (WT) and *sfr8* leaf discs when they were exposed to -5°C, -7°C and -9°C. Leaves were excised from plants after 5 weeks of growth at 20°C with and without an additional 2 weeks of cold-acclimation at 5°C. **A)** Both acclimated and non-acclimated WT and *sfr8* plants with 6 replicate tubes for each genotype, treatment and temperature. Results were arcsine transformed and analysed via one-way ANOVA. **B)** Acclimated plants and **C)** non-acclimated plants show the average results of 3 biological replicate experiments with 6 replicate tubes per genotype and temperature, each containing 3 leaf discs. Results were arcsine transformed and analysed by least-squares means (LSM) comparison at each temperature (one-way ANOVA/LSM ***, *P* <0.001, **, *P* <0.01, *, *P* <0.05). Error bars represent +/- 1 SE calculated from arcsine-transformed data.

3.1.4 RGII dimerisation mutants

mur1 mutants are defective in their ability to synthesise L-fucose (Bonin *et al.*, 1997). Fucose is normally present in RGII side chain A which is involved in the borate diester covalent linkage of RGII monomers via its apiose residues (Kobayashi, Matoh and Azuma, 1996). The lack of fucose in side chain A results in truncation which affects bond formation and, therefore, the ability of RGII to dimerise (Pabst *et al.*, 2013). *sfr8* is another *MUR1* mutant which also shows reduced RGII dimerisation due to truncation of side chain A (Table 1.1 and Figure 3.4). Both *mur1-1* and *sfr8* alleles were investigated to ensure the response to fucose supplementation was consistant. Both mutants were shown to have increased freezing sensitivity when compared to WT, however, spraying these mutants with 60 ml of 10 mM fucose once a week restored freezing sensitivity to WT-like levels (Panter, 2019). This suggests that the freezing sensitivity seen in these mutants is linked to defective fucosylation. Further investigation was carried out to determine the effect of fucose supplementation on RGII dimerisation. Dimerisation levels in supplemented and un-supplemented WT, *sfr8* and *mur1-1* plants were investigated (Figure 3.4) and it was seen that spraying *sfr8* and *mur1-1* with 60 ml of 10 mM fucose once a week restored RGII dimerisation to WT-like levels. This suggests that the restoration of RGII dimerisation by fucose supplementation may be linked to the recovered freezing tolerance seen upon supplementation.

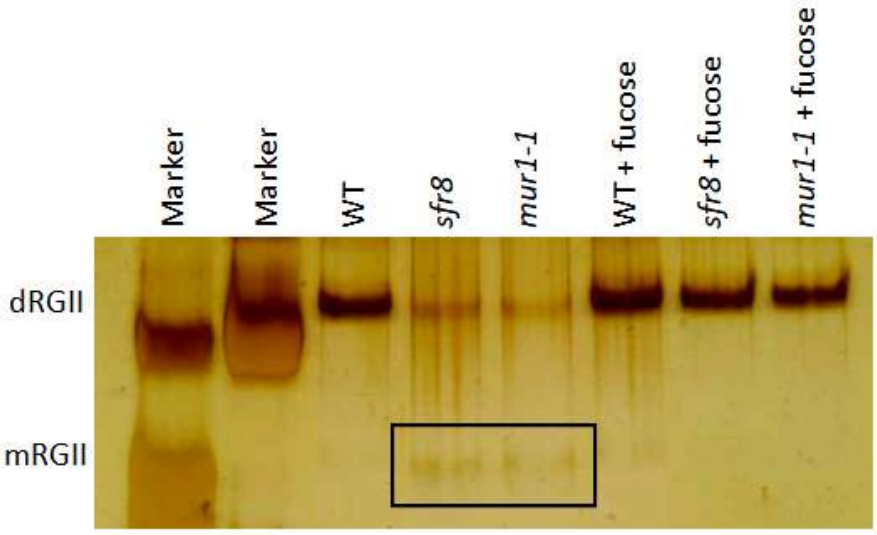

Figure 3.4: RGII dimerisation levels of 5-week-old *sfr8, mur1-1* and WT plants with and without fucose supplementation. RGII dimerisation levels of 5-week-old Col-0 (WT) *mur1-1* and *sfr8* plants with and without fucose supplementation were examined via PAGE analysis. Cell wall extracts were digested with EPG and run on a polyacrylamide gel. The gel was stained with silver nitrate and purified RGII standards were used as size markers. Black box highlights monomeric RGII bands.

Supplementation of *mur1-1* mutants with BA was shown to restore WT-like phenotype, cell wall strength and RGII dimerisation levels (O'Neill *et al.*, 2001; Ryden *et al.*, 2003). Panter *et al*.

(2019) also showed the ability of BA supplementation to restore freezing sensitivity to WT levels, however, RGII dimerisation levels did not appear to be restored. This is thought to be due to the fact that supplementation with BA does not address the inability of *sfr8* mutants to synthesize L-fucose. This means the side chains involved in RGII dimerisation continue to lack their L-fucose residue which results in truncation of the side chains (Pabst *et al.*, 2013). This affects the stability of the dimer bond (O'Neill *et al.*, 2001) so while dimerisation may be restored with supplementation, the fragile borate diester linkage may not be able to survive the AIR extraction process required to assess dimerisation (Stephen Fry, personal communication). As a result, it is unclear whether the reason dimerised RGII is not observed is because none is present or because the bonds are just too weak to survive the AIR extraction process.

3.1.5 Characterisation and dimerisation status of *bor* mutants

To confirm whether a lack of RGII dimerisation is the cause of freezing sensitivity in *sfr8* other mutants, defective in RGII dimerisation via an alternative mechanism, were investigated. Mutants with genes encoding boron transporter proteins; BOR1 (*bor1-3*), BOR2 (*bor2-1*, *bor2-2*) or both BOR1 and BOR2 (*bor1-3/2-1*) disrupted were examined (Alonso *et al.*, 2003; Rosso *et al.*, 2003). Reduced RGII dimerisation levels have previously been seen in the roots of *BOR1* and *BOR2* mutants (Miwa *et al.*, 2013). Given the requirement for boron for dimerisation of RGII in the roots it was thought that these mutants would also be unable to dimerise RGII in their leaves. Characterisation of *bor* mutants was carried out to determine if these mutants were an appropriate tool to investigate the effects of RGII dimerisation in leaves.

Growth habit (Figure 3.5A) was examined first as most experiments on *bor* mutants have focused on root characterisation (Miwa *et al.*, 2013). Both *bor2-1* and *bor2-2* mutants displayed a leaf size and shape very similar to that observed in WT plants. In comparison *bor1-3* plants were stunted with reduced leaf size and curling of the leaves. This phenotype was seen to an even greater degree in the *bor1-3/2-1* double mutant with the plants appearing very stunted.

Leaf RGII dimerisation levels were assessed in the *bor* mutants to determine their suitability for these experiments. Dimerisation levels have been shown to be reduced in the roots of *BOR1* and *BOR2* mutants (Miwa *et al.*, 2013). However, most freezing damage occurs in the leaves as the roots are protected underground therefore, RGII dimerisation levels in the leaves would be predicted to be far more relevant with respect to freezing tolerance. To determine the level of RGII dimerisation occurring in *bor* mutant leaves the alcohol insoluble cell wall residue (AIR) was extracted from the leaves of three plants per genotype and digested with Endo-polygalacturonase (EPG). EPG releases the pectin domains of RGII and, when separated on a

poly-acrylamide gel, allows differentiation between the monomeric and dimeric forms based on size. Dimeric RGII shows reduced mobility when moving through the gel so appears as a distinct, higher molecular weight band compared to monomeric RGII which is able to migrate through the gel more quickly.

An RGII dimer band was present on all samples but both *bor1-3* and *bor1-3/2-1* mutants also showed a monomer band (Figure 3.5B). The presence of monomeric RGII indicates a reduction in dimeric RGII. This showed these mutants were defective in their ability to dimerise RGII and are therefore suitable to address the question of how reduced RGII dimerisation affects freezing tolerance.

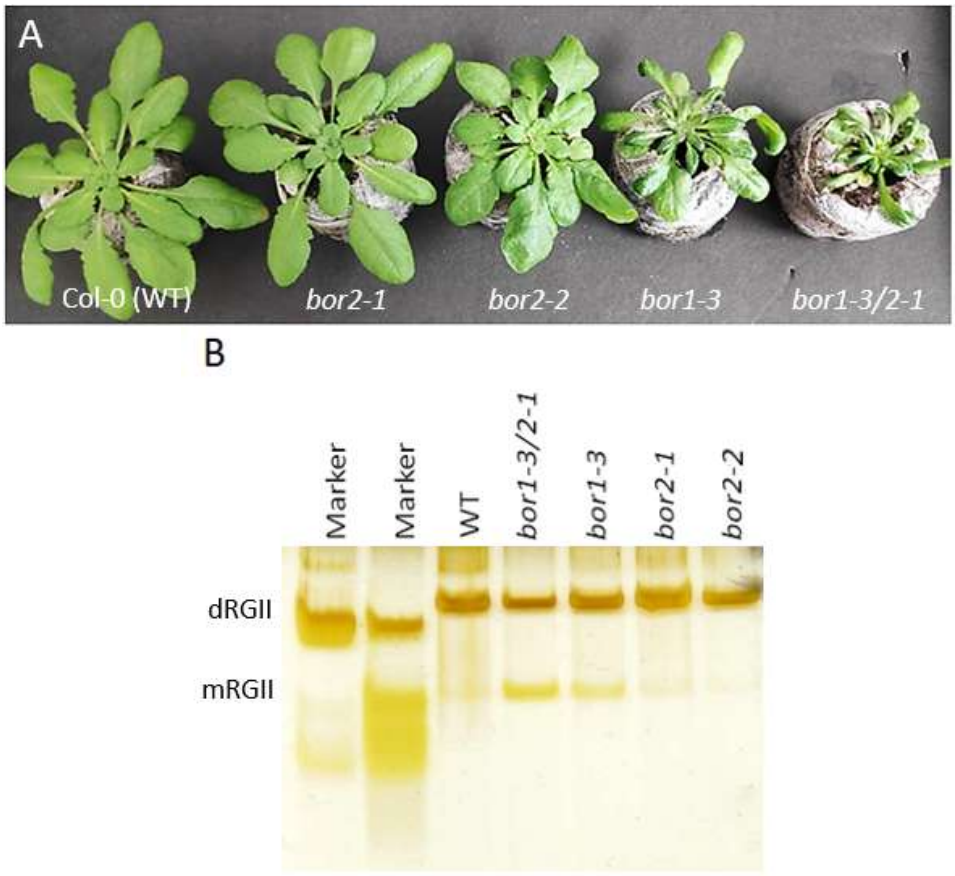

Figure 3.5: Phenotypic assessment and RGII dimerisation status of *bor* mutant plants. **A)** Assessment of gross morphology of 5-week-old Col-0 (WT) and *bor* mutant plants. **B)** RGII dimerisation levels in leaf cell wall extracts were examined via PAGE analysis. Cell wall extracts from 5-week-old Col-0 (WT)*, bor2-1, bor1-3* and *bor1-3/2-1* leaves were digested with EPG and run on a polyacrylamide gel. The gel was stained with silver nitrate and purified RGII standards used as size markers.

3.1.6 Freezing sensitivity in RGII-dimerisation *bor* mutants

Reduced RGII dimerisation observed in some *bor* mutant lines allowed them to be used to address the link between RGII dimerisation and freezing tolerance. To determine if defective

RGII dimerisation is responsible for the freezing-sensitive phenotype observed in *sfr8* plants, an EL assay was carried out on the *bor* mutants.

An EL assay was carried out on all *bor* mutant lines at two temperatures. Due to the small leaf size of both *bor1-3* and *bor1-3/2-1* mutants, whole leaves were used rather than leaf discs. WT and mutant leaves were matched both for size (Figure 3.6A) and for age (Figure 3.6B). In both experiments *bor2-1* and *bor2-2* plants behaved in the same way. *bor2-2* was excluded from the results displayed here due to its lack of RGII dimerisation phenotype and the similarity between *bor2-1 and bor2-2* mutants. The results of experiments with *bor2-2* can be seen in Appendix A. When matched for age, *bor2-1* (Figure 3.6B) showed leakage levels very similar to those observed in WT. However, when matched for size (Figure 3.6A) *bor2-1* showed significantly lower levels of leakage (P <0.01 at -4°C and P <0.05 at -6°C) than WT leaves. In contrast, *bor1-3* displayed significantly higher levels of leakage in comparison to WT when matched for age (P <0.001) and when matched for size (P <0.001 at -4°C and P <0.01 at -6°C). These results suggest that while *bor2-1* may be less freezing sensitive than WT, *bor1-3* is much more freezing sensitive than WT. This indicates that the proteins encoded by these genes may play very different roles in freezing tolerance.

bor1-3/2-1 plants were seen to display significantly higher levels of EL than WT (P <0.001) in both experiments at both temperatures. *bor1-3/2-1* also displayed significantly higher levels of leakage than *bor1-3* at -4°C in both experiments (P <0.01 and P <0.001 respectively). At -6°C the difference between *bor1-3* and *bor1-3/2-1* was less significant (P <0.05 in Figure 3.6A)), however, this could be caused by *bor1-3/2-1* reaching the maximum level of leakage possible resulting in the difference appearing less significant. The similarity observed between these sets of results indicates that these are an accurate depiction of the damage caused by freezing to each of these mutants and that these results are not the outcome of variation in the size or age of leaves used.

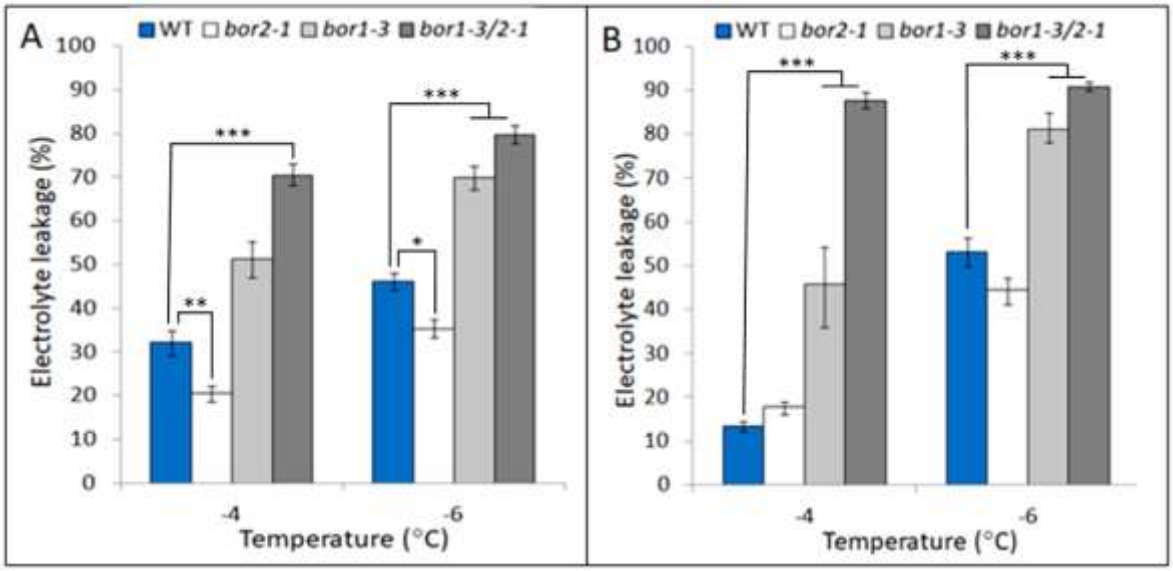

Figure 3.6: Electrolyte leakage from whole leaves of WT and *bor* mutant plants. Col-0 (WT), *bor2-1, bor1-3* and *bor1-3/2-1* plants were grown for 5 weeks then 3 leaves were excised from each plant. Values indicate percentage of electrolytes lost from leaves when they were exposed to -4°C and -5°C. Each bar represents the average taken from 6 replicate tubes for each genotype and temperature. Error bars show +/- 1 SE from arcsine transformed data. **A)** Leaves were matched for size across all genotypes. **B)** Leaves used were matched for age across all genotypes by selecting leaves numbered 10, 11 and 12 from each plant used (see Figure 3.7A). Results were arcsine transformed and analysed by least-squares means (LSM) comparison at each temperature (one-way ANOVA/LSM ***, *P* <0.001, **, *P* <0.01, *, *P* <0.05). Error bars represent +/- 1 SE calculated from arcsine-transformed data.

3.1.7 Further investigation into the effect of leaf size and age on freezing sensitivity

To establish a better understanding of the effect of leaf size on freezing tolerance, an EL experiment was carried out. The relationship between leaf size and age can be seen in Figure 3.7A. Medium aged, large leaves (10, 11 and 12) would normally be chosen for an EL assay. These leaves were compared against small, juvenile leaves (1, 2 and 3) and small, young leaves (15, 16 and 17) to determine the effect age and size has on freezing sensitivity. Whole leaves were used from 5-week-old WT plants with 6 biological replicates for each age and temperature (Figure 3.7B). To ensure a similar amount of tissue was used for each age tested 3 leaves per rep were used for small, juvenile and small, young leaves whereas one leaf was used for medium aged, large leaves. While higher levels of variation were seen in the medium aged large leaves, they showed less leakage on average than either very young or juvenile leaves. These results would suggest that small leaves, whether young or juvenile, were more sensitive to freezing – which may be a consequence of their greater surface area to volume ratio.

However, there are some problems with this comparison as leaves of different ages have different morphology which may impact their freezing sensitivity. Juvenile leaves are the first leaves produced by the plant and have several differences to mature leaves: they are smaller, more round in shape and have fewer trichomes. Similarly young leaves may respond differently to older leaves as they are still undergoing active growth which requires deposition of cell wall material which may impact freezing tolerance. These morphological differences may be impacting freezing sensitivity. While further comparisons would be needed to definitively determine the effect of age on freezing sensitivity, these results may suggest that size has more influence than age on freezing sensitivity.

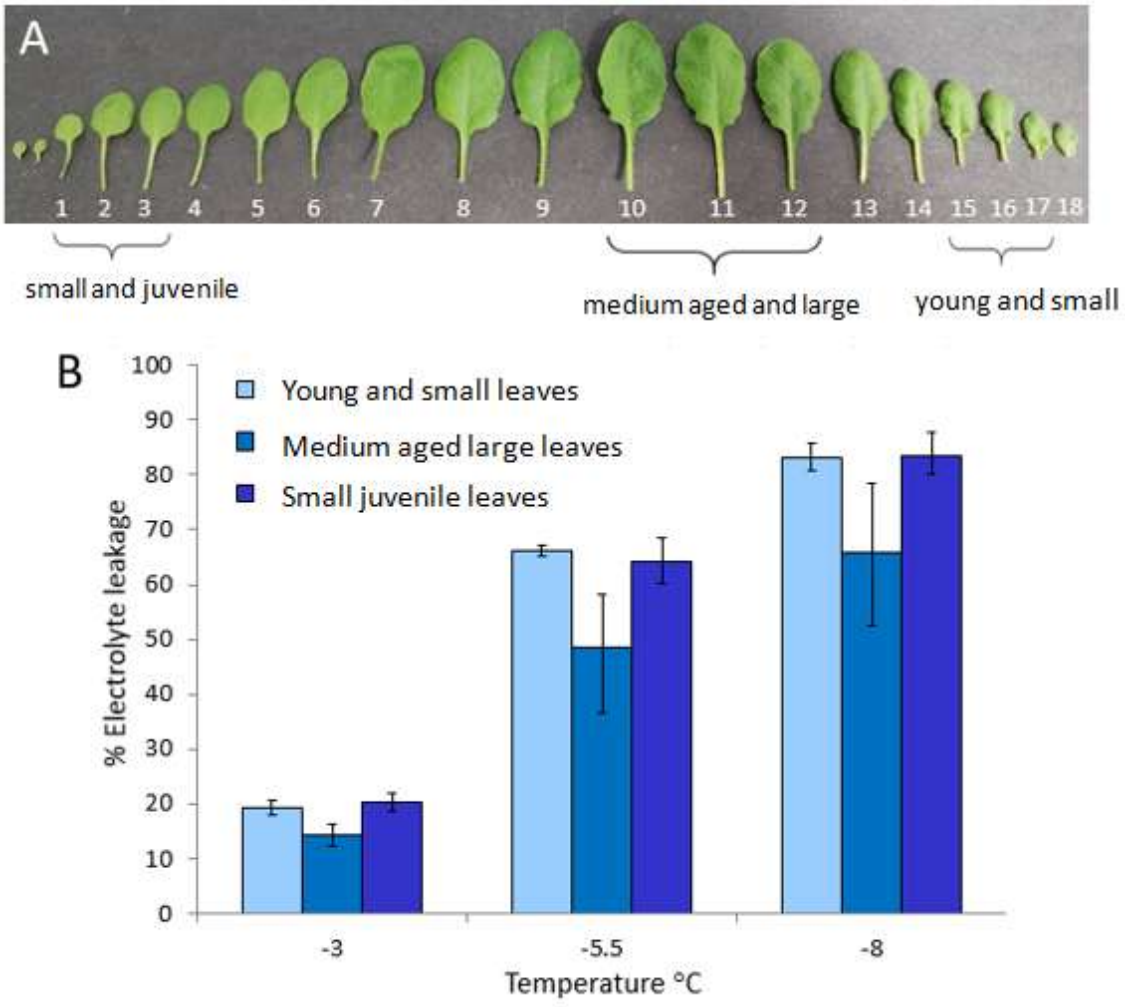

Figure 3.7: Relationship between leaf size and age and impact this has on freezing tolerance. **A)** Size and age relationship of Col-0 (WT) leaves. Leaf groups for EL highlighted **B)** EL using whole leaves comparing medium aged, large leaves (10, 11 and 12) to young and small (15, 16 and 17) and juvenile and small (1, 2, and 3) leaves. Values indicate percentage of electrolytes lost from leaves when exposed to -3°C, -5.5°C and -8°C. 6 replicate tubes were used for each leaf group per experiment. Error bars represent +/- 1 SE calculated from arcsine-transformed data.

This experiment can be related to the results shown in Figure 3.6 where leaves were matched for size and age. In Figure 3.6B leaves were matched for age. Therefore, small *bor1-3* and *bor1-3/2-1* leaves were compared to medium WT and *bor2-1* leaves. Since the results above suggest that smaller leaves have greater sensitivity to freezing damage, the difference in freezing sensitivity between WT and *bor1-3/2-1* and *bor1-3* mutants may be overrepresented in Figure 3.6B. However, when matched for size (Figure 3.6A) medium-aged *bor1-3* and *bor1-3/2-1* mutant leaves had to be compared to younger WT and *bor2-1* mutant leaves. As the results above suggest that younger leaves may also be more sensitive to freezing, WT leaves may appear more sensitive to freezing due to their young age. This means, if anything, the difference in freezing tolerance observed between WT and *bor1-3* and *bor1-3/2-1* mutants is underrepresented in Figure 3.6A and a significant difference (P <0.001) can still be seen between *bor1-3/2-1 bor1-3* and WT leaves in this experiment.

Triplicate data was used to ensure the accuracy of the *bor* experiment: the two experiments shown in Figure 3.6 were combined along with an additional experiment also using size-matched leaves (Figure 3.8). Size matched leaves were used twice as this is least likely to over represent any differences between the data. When combined, the triplicate results showed that *bor1-3* and *bor1-3/2-1* mutants both show significantly higher levels of leakage (P <0.001), and therefore damage, than WT (Figure 3.8). This indicates that *bor1-3* and *bor1-3/2-1* are significantly more sensitive to freezing that WT. However, as seen in the single experiments, *bor1-3/2-1* had significantly higher levels of leakage (P <0.001) than *bor1-3*. *bor2-1* showed WT levels of freezing sensitivity, consistent with its WT-like pattern of RGII dimerisation in leaf tissue. This indicates that there is a link between RGII dimerisation levels and freezing sensitivity.

These results show that mutants with *BOR1* knocked out always display significantly higher levels of leakage, and therefore freezing sensitivity, than WT. These mutants also showed reduced dimerisation indicating there might be a link between RGII dimerisation and freezing tolerance. However, it was also observed that loss of *BOR1* and *BOR2* in combination increased the leakage levels to significantly higher than those seen in single mutants. This suggests lacking the combination of both BOR1 and BOR2 proteins has a cumulative effect resulting in significantly higher levels of freezing sensitivity.

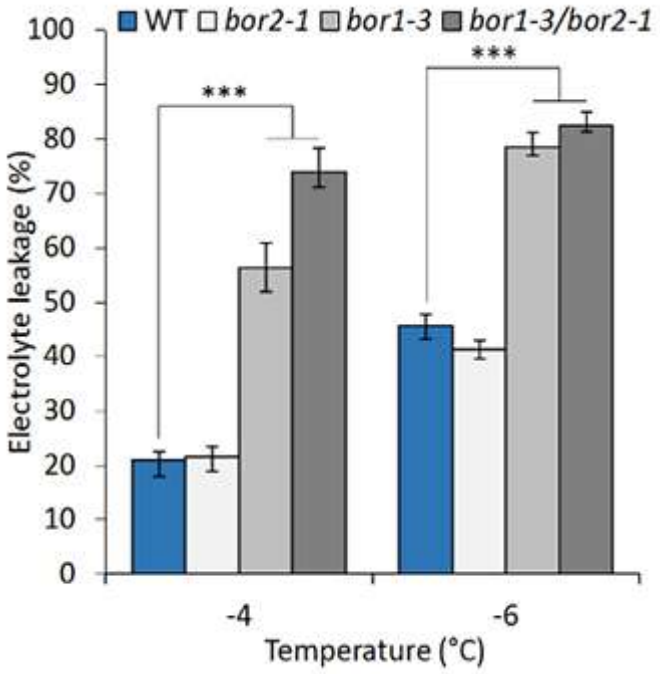

Figure 3.8: Electrolyte leakage levels from whole leaves of WT and *bor* mutant plants. Leaves were excised from 5-week-old Col-0 (WT), *bor2-1, bor1-3* and *bor1-3/2-1* plants and frozen. Values indicate percentage of electrolytes lost from leaves when exposed to -4°C and -6°C. Results are an average of 3 biological replicate experiments and 6 replicate tubes, each containing 3 leaves, were used for each genotype and temperature per experiment. Results were arcsine transformed and analysed by least-squares means (LSM) comparison at each temperature (one-way ANOVA/LSM ***, *P* <0.001). Error bars represent +/- 1 SE calculated from arcsine-transformed data.

3.1.8 Phenotypic assessment of RGII dimerisation mutants when supplemented with boric acid (BA)

bor1-3/2-1 mutants were investigated to confirm whether supplementation could restore RGII dimerisation levels and WT freezing tolerance in these dimerisation mutants. Both supplemented and un-supplemented WT plants were used as controls to ensure any results caused by supplementation were exclusive to the dimerisation mutants.

Plant growth via hydroponics was investigated as a possible replacement for growing plants on peat plugs. It was thought that this might ensure even distribution of nutrients to the plants when supplementing with additional BA. Plants were germinated in agar plugs and then grown in sterile plastic tubs containing approximately 1.5 L liquid MS. The roots of each plant were contained within a modified 20-ml centrifuge tube to ensure the plants remained separate for experimentation.

Stunted root growth as characterised in (Miwa et al. 2013) was observed in *bor1-3/2-1* seedlings which made the transition from agar growth media to liquid media more challenging and increased the death rate because roots were too short to reach the media. In plants grown in this hydroponic system, the stunted mutant phenotype observed in both the *bor1-3* and *bor1-3/2-1* mutants was reduced. This suggests that, in the hydroponic set up, ½ x MS media contains enough BA to improve the phenotype in the mutant lines. This indicates that the hydroponic set up is a very efficient delivery system for supplementing plants with boron. While this growth system does appear to improve the BA delivery system it would be hard to test un-supplemented plants for comparison given how readily plants take up BA in this system. Recovery of WT like growth in mutant lines was seen using media containing as little as ¼ x MS (Figure 3.9).

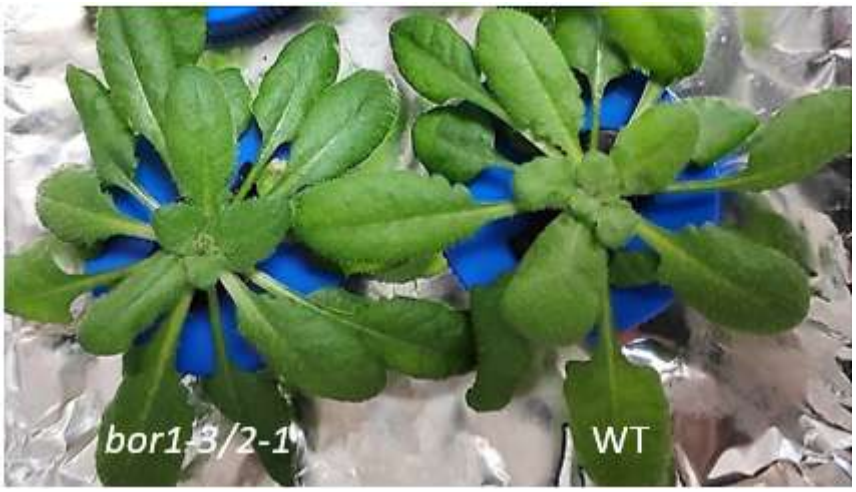

Figure 3.9: Assessment of gross morphology in 5-week-old *bor1-3/2-1* and WT plants grown in a hydroponic set up. Col-0 (WT) and *bor1-3/2-1* plants were germinated on agar and grown in ¼ x MS liquid media.

The growth habit of *bor1-3/2-1* mutant plants grown on peat plugs with and without supplementation was also assessed. Plants were germinated on ½ x MS media supplemented with 1 mM BA and then watered with 20 mg/L BA. At 5 weeks growth, phenotypic characterisation (Figure 3.10A) showed that when supplemented, *bor1-3/2-1* mutants showed restored WT phenotype. WT plants did not appear to be affected by supplementation. In light of these results and the challenges involved in hydroponic growth this system was not pursued further.

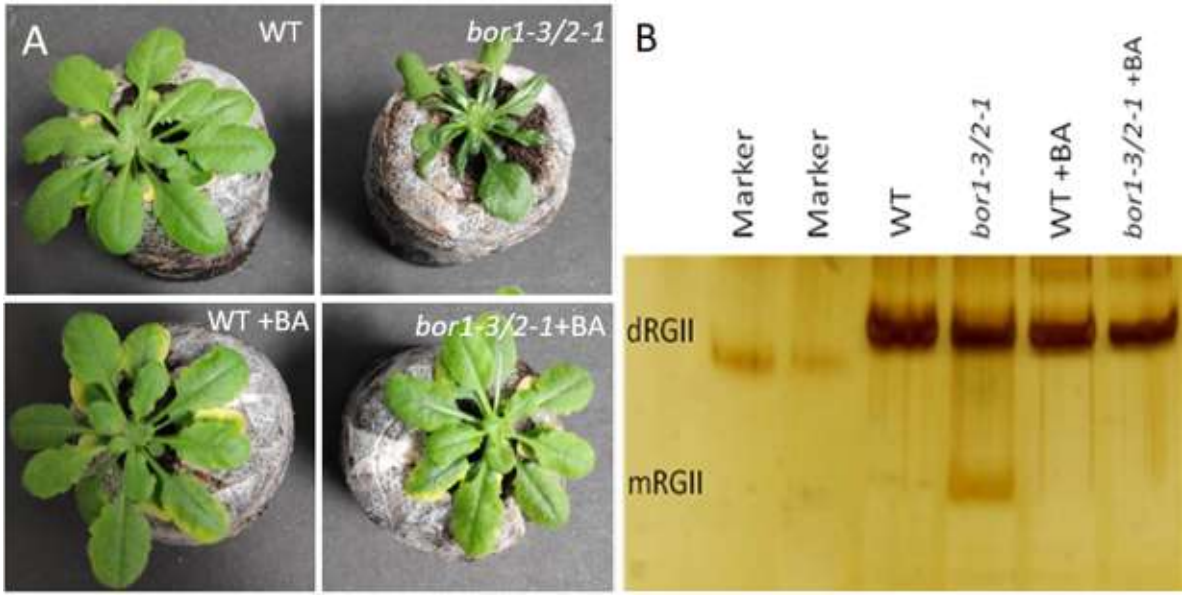

Figure 3.10: Assessment of gross morphology and RGII dimerisation levels of 5-week-old WT and *bor1-3/2-1* plants with and without supplementation with BA. A) Assessment of gross morphology in Col-0 (WT) and *bor1-3/2-1* plants with and without supplementation with 20 mg/L BA. **B)** RGII dimerisation levels in leaf cell wall extracts were examined via PAGE analysis. Cell wall extracts from 5-week-old Col-0 (WT) and *bor1-3/2-1* leaves with and without BA supplementation were digested with EpG and run on a polyacrylamide gel. The gel was stained with silver nitrate and purified RGII standards were used as size markers.

3.1.9 RGII dimerisation in supplemented and un-supplemented *bor* mutant plants

In order to assess the effect of supplementing plants with BA on RGII dimerisation a poly acrylamide gel assay was used. WT and *bor1-3/2-1* plants watered with or without 20 mg/L BA were compared. As seen in Figure 3.5B, a monomeric RGII band is present in the un-supplemented *bor1-3/2-1* mutant but not in un-supplemented WT (Figure 3.10B). When plants were supplemented with BA this band was no longer present indicating restoration of RGII dimerisation. Dimerisation levels in supplemented WT plants remained unchanged. These results indicate that supplementing with BA only affects mutants with decreased RGII dimerisation as dimerisation was only affected in those mutants. Restoration of the stunted *bor1-3/2-1* phenotype upon supplementation (Figure 3.10B) would suggest this phenotype is due to boron deficiency and therefore potentially a lack of RGII dimerisation.

3.1.10 Freezing sensitivity in supplemented and un-supplemented *bor* mutant plants

To investigate whether BA supplementation restores freezing tolerance in *bor* mutants, an EL assay was performed on size-matched supplemented and un-supplemented WT and *bor1-3/2-1* leaves (Figure 3.11). Again, no difference was observed between supplemented and un-supplemented WT plants showing that in WT plants additional boron has no effect on freezing sensitivity. Un-supplemented *bor1-3/2-1* leaves showed significantly higher levels of leakage and damage (P <0.001) than WT leaves at all temperatures. This confirmed that, as previously observed (Figures 3.6 and 3.8) *bor1-3/2-1* plants are much more freezing sensitive than WT plants. Supplementation of *bor1-3/2-1* plants with BA reduced leakage levels of the mutant leaves to levels similar to those seen in supplemented WT (P <0.5 at all temperatures). When supplemented *bor1-3/2-1* was compared to un-supplemented *bor1-3/2-1* the freezing sensitivity was seen to be significantly reduced (P <0.001 at -3 and -5°C) and while a smaller reduction was observed at -8°C this was still significant (P <0.05). This indicates that supplementation with additional boron during growth restored the freezing sensitivity of the *bor1-3/2-1* mutant to WT-like levels. It may be harder to observe differences between plants at lower temperatures since all plants are approaching maximum (100%) electrolyte leakage at these temperatures due to the damage sustained by freezing.

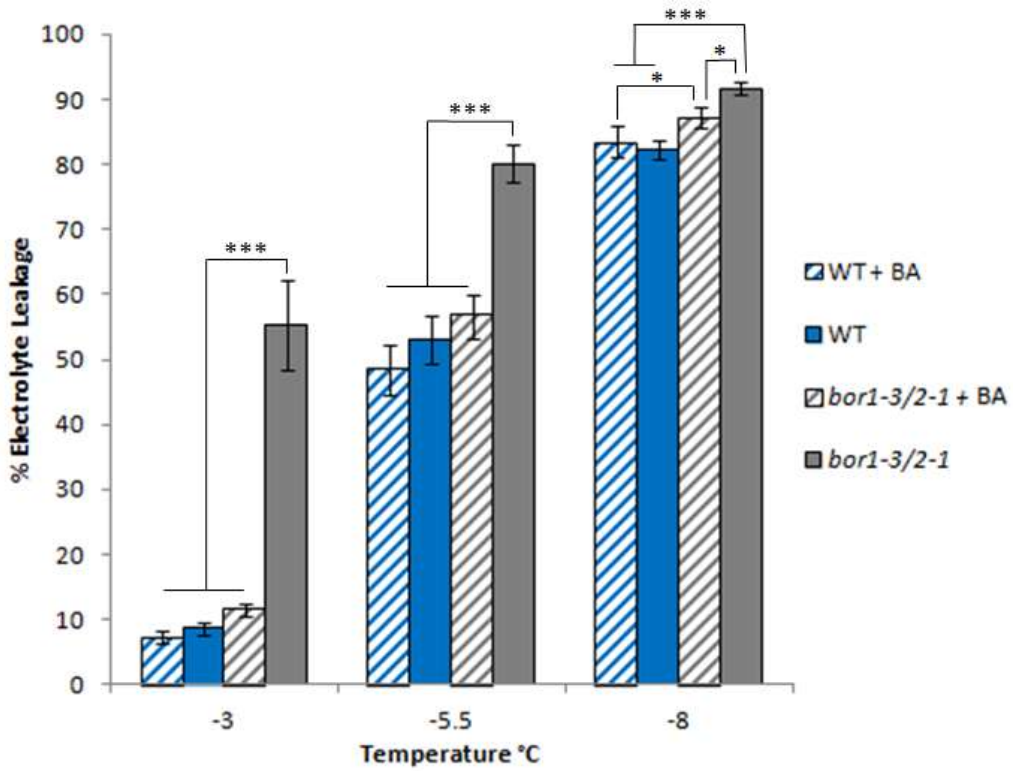

Figure 3.11: Electrolyte leakage levels on whole WT and *bor1-3/2-1* leaves with and without BA supplementation. Col-0 (WT) and *bor1-3/2-1* supplemented plants were grown on 1 mM BA and then watered with 20 mg/L BA. Leaves were excised from 5-week-old plants and frozen, values indicate percentage of electrolytes lost from leaves when exposed to -3°C, -5.5°C and -8°C. Results are an average of 3 biological replicate experiments and 6 replicate tubes, each containing 3 leaves, were used for each

genotype, treatment and temperature per experiment. Results were arcsine transformed and analysed by least-squares means (LSM) comparison at each temperature (one-way ANOVA/LSM ***, *P* <0.001). Error bars represent +/- 1 SE calculated from arcsine-transformed data.

Restoration of both RGII dimerisation and freezing sensitivity in supplemented *bor1-3/2-1* plants is similar to the restoration observed in supplemented *sfr8*. This, along with the link observed between the presence of monomeric RGII and increased freezing sensitivity indicates that RGII dimerisation is likely to be involved in freezing sensitivity.

3.2 RGII dimerisation and desiccation

3.2.1 Desiccation in *sfr8* mutant leaves

sfr8 plants have been shown to display an altered desiccation phenotype and dry more rapidly than WT when exposed to drought conditions (Panter, 2019). *sfr8* plants supplemented with BA were investigated via leaf drying assay to determine if this phenotype was the result of defective RGII dimerisation. Single leaves were excised from 5-week-old *sfr8* and WT plants grown with or without 20 mg/L BA. Leaves were placed abaxial side up at room temperature and weighed every hour for 8 h and then again at 48 h and after 7 days. Seven leaves were used per genotype and treatment and an average is shown. The decrease in mass was used as a measure of water loss to determine the rate of drying.

Two replicates of the same experiment (Figures 3.12 A and B) are shown because the results showed some variation between experiments. Both experiments showed that *sfr8* leaves lost water much faster than WT leaves. However, Figure 3.12A shows that there was a very fast initial rate of water loss with leaves reaching almost complete desiccation after only 2 h, in contrast Figure 3.12 B showed a much steadier rate of water loss with almost complete dryness occurring after 5 h. In both experiments it was found that supplementing *sfr8* with BA decreased the rate of water loss. The rate of water loss was shown to be partially (Figure 3.12B) or fully (Figure 3.12A) restored to WT-like rates when supplemented. The most notable difference between the experiments was the effect of BA supplementation on WT leaves. Figure 3.12A shows that BA supplementation caused an increase in water loss in WT plants whereas Figure 3.12B shows that supplementation caused a decrease in water loss. Although variation in the results of BA supplementation on WT leaves was of concern, since both experiments showed increased water loss in *sfr8* leaves and some degree of restoration with BA supplementation, RGII dimerisation was postulated to play a role in desiccation.

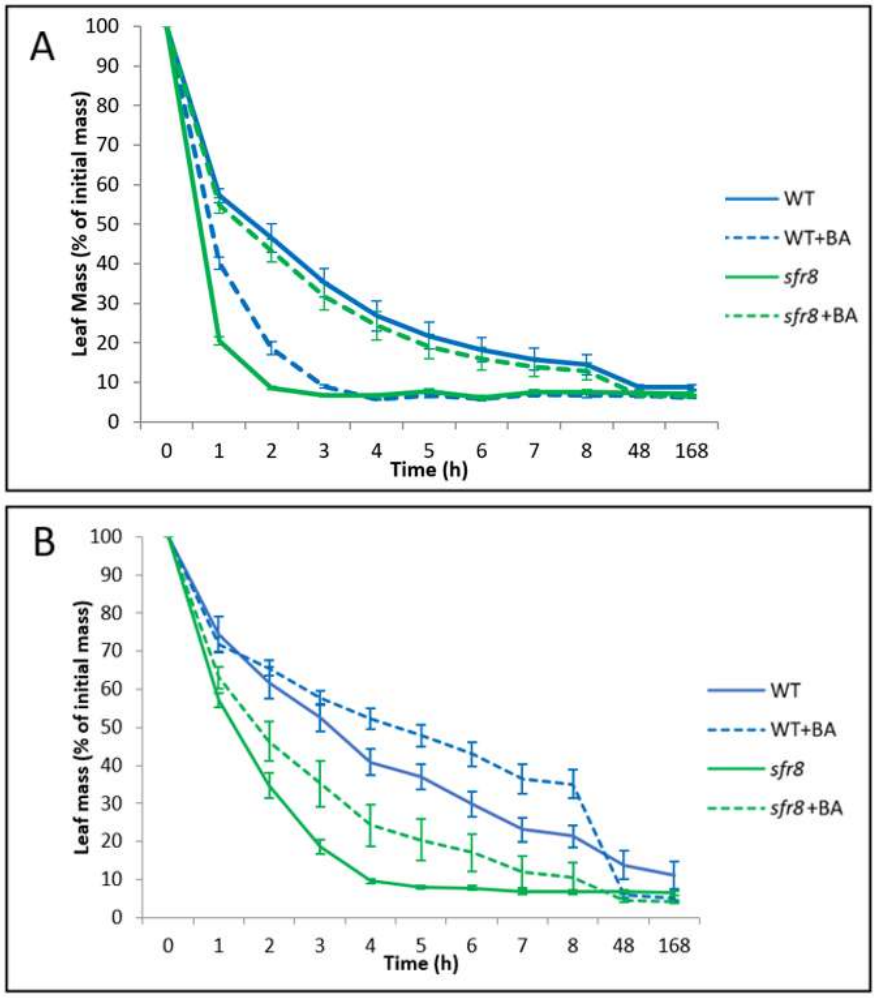

Figure 3.12: Water loss from WT and *sfr8* leaves with without supplementation with BA. A) Rep one **B)** Rep two. Leaves were excised from 5-week-old *sfr8* and Col-0 (WT) plants and weighed every hour for 8 h and then at 48 h and 7 days. The rate of mass decrease over time equates to the rate of water loss. Error bars show +/- 1SE.

3.2.2 Desiccation in *bor* mutants

Dimerisation mutant *bor1-3/2-1* was investigated to further determine the effect of RGII dimerisation on desiccation. A leaf drying assay was carried out on leaves excised from 5-week-old WT and *bor1-3/2-1* plants which were watered either with or without additional BA (Figure 3.13). Leaves were weighed every hour and then additional weights were taken at 48 h and 7 days. Un-supplemented *bor1-3/2-1* showed higher rates of water loss than WT. In comparison, supplemented *bor1-3/2-1* showed water loss at rates similar to those observed in WT. In this experiment BA supplementation in WT plants did not appear to have an effect.

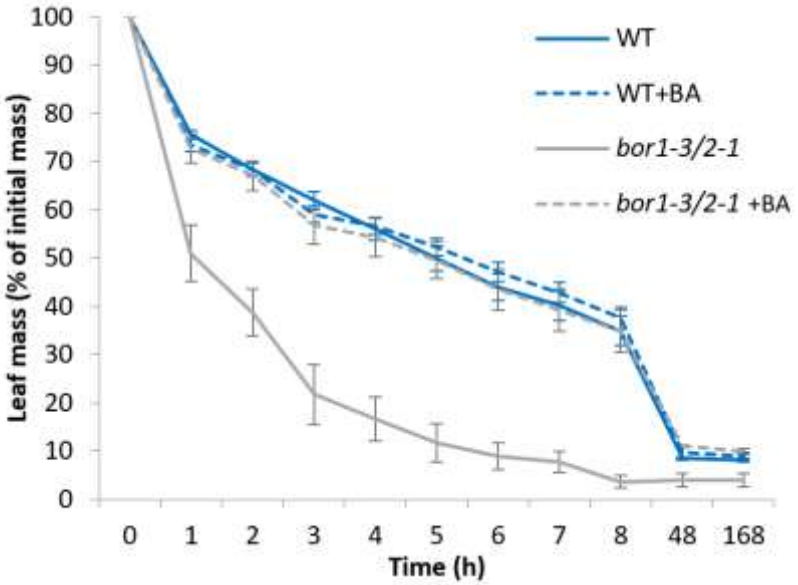

Figure 3.13: Water loss from WT and *bor1-3/2-1* mutant leaves with and without BA supplementation. Leaves were excised from 5-week-old Col-0 (WT) and *bor1-3/2-1* plants. Due to the small size of un-supplemented *bor1-3/2-1* leaves, 3 leaves were used per rep with 7 reps per genotype and treatment. Leaves were weighed every hour for 8 h and then at 48 h and 7 days. The rate of mass decrease over time equates to the rate of water loss from the leaves. Error bars show +/- 1SE.

These results suggested that supplementing *bor1-3/2-1* with BA can restore the WT desiccation phenotype. However, it is possible that this result is affected by the small leaf size observed in un-supplemented *bor1-3/2-1* mutants rather than by a change in RGII dimerisation. The decreased size of these mutants, and therefore lower surface area to volume ratio, may result in faster drying. To investigate this further a thermal imaging experiment was carried out. This allows the whole plant to be monitored and water loss and guard cell dynamics to be compared.

3.2.3 Thermal imaging of *bor1-3/2-1* and WT plants under drought conditions

Thermal imaging of plants measures stomatal conductance and can be used as a measure of the rate of water loss occurring in the leaves (Merlot *et al.*, 2002). The majority of water loss in plants occurs through the stomata. When water is lost through the stomata it evaporates from the leaf surface, this causes a reduction in leaf temperature as evaporative cooling takes place (Jones, 1999). During drought conditions plants normally close their stomata and an increase in temperature is observed as less evaporative cooling takes place. The rate of water loss can be used to infer stomatal response to drought conditions (Wang *et al.*, 2004).

Thermal imaging of *sfr8* has been carried out by Panter (2019) and suggests that *sfr8* plants have an altered response to desiccation when compared with WT. To further investigate the role of RGII dimerisation in desiccation thermal imaging of *bor1-3/2-1* was carried out. 5-week-old WT and *bor1-3/2-1* plants (Figure 3.14D) were moved from 100% humidity to 50% humidity and watering was stopped. Thermal images were taken every 30 s for 4 days to compare the rate of water loss via evaporative cooling in these two lines under drought conditions.

The first image (Figure 3.14A) was taken after a 40-min period to allow plants to adjust to the change in temperature and humidity. It was observed that *bor1-3/2-1* plants were cooler than WT plants, indicating higher levels of evaporative cooling were occurring. After approximately 24 h (Figure 3.14B) while *bor1-3/2-1* plants remained cooler than WT plants both genotypes were seen to have increased their temperature by a similar degree. This suggested that the plants were responding to the drought conditions by closing their stomata to reduce water loss. However, *bor1-3/2-1* plants remained cooler than WT suggesting they were still undergoing a higher level of evaporative cooling. The increased water loss observed in *bor1-3/2-1* mutants could be due to a number of causes. These plants may be slower to respond to external stimuli, have stomata that are defective in their ability to respond to external stimuli or might have a higher density of stomata. It is also possible that they simply did not run out of water so quickly as the *bor1-3/2-1* plants were significantly smaller than WT but on the same size of peat plug. Both *bor1-3/2-1* and WT plants maintained their temperatures between 24 and 72 h and no temperature increases were observed in either genotype (Figure 3.14C). This suggested that the mutant responds to the drought stimulus in a similar way and at a similar rate to WT plants. The cooler temperature of *bor1-3/2-1* observed throughout the experiment suggested that it was continually undergoing a higher level of evaporative cooling. This is most likely to be caused either by the plant having stomata that cannot effectively respond to drought conditions by closing or by having a higher density of stomata, however, the size of the plants may also be having an effect as mentioned above. Given the size related complications this experiment was not pursued further and *bor1-3* and *bor2-1* were not investigated.

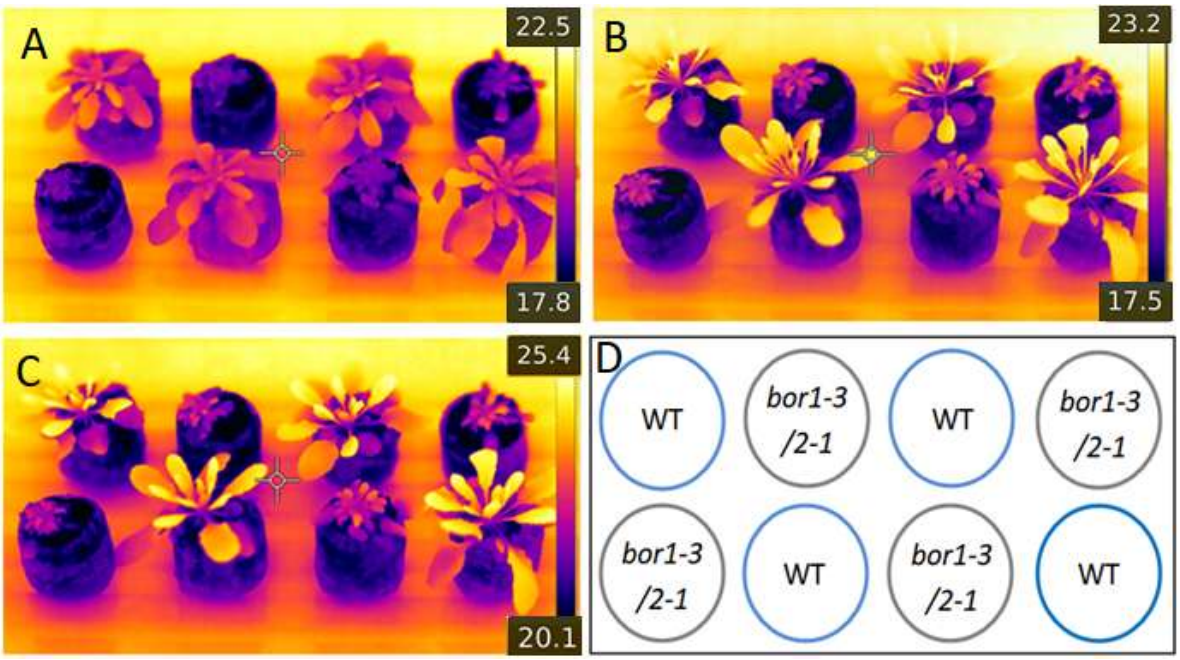

Figure 3.14: Thermal imaging of 5-week old WT and *bor1-3/2-1* plants. Infrared thermography images have a temperature colour scale on the right and show Col-0 (WT) and *bor1-3/2-1* plants approximately **A)** 40 min after moving from 100% to 50% humidity, **B)** 24 h after transfer, **C)** 72h after transfer. **D)** Arrangement of WT and *bor1-3/2-1* plants. Emissivity was set at 0.95, ambient temperature at 23°C and humidity at 50%.

3.2.4 Stomatal morphology in RGII dimerisation mutants

The *MUR1* mutant, *sfr8,* has been shown to have altered desiccation (Figure 3.12). This was also observed by Panter (2019). Zhang *et al.* (2011) identified a series of mutants with *increased susceptibility to COR-deficient* Pst *DC3000* (*scord*). One of these mutants, *scord6* is another mutant allele of MUR1 and displayed an altered stomatal morphology. Guard cells normally have an extended ledge or lip that forms around the edge of the stomatal pore known as the cuticular ledge. SEM and TEM imaging shows *scord6* mutants have a collapsed or absent cuticular ledge on their stomata (Zhang *et al.*, 2011). It is hypothesised that this might affect the ability of the stomata to open and close. As *sfr8* is also mutated in its *MUR1* gene, altered stomatal morphology may be the cause of the increased desiccation rates observed. The *bor1-3/2-1* plants also showed more rapid desiccation and *bor1-3/2-1, bor1-3* and *sfr8* all have defective RGII dimerisation. Therefore, to investigate whether RGII dimerisation was involved in stomatal morphology stomata from both *sfr8* and *bor* mutants were investigated and compared to WT plants.

For SEM imaging, leaf sections were fixed in Karnovsky's fixative and osmium tetroxide and dehydrated via an ethanol series. Sections were then dried at the critical point, mounted on silicon chips and coated with ~10 nm platinum to be imaged in a S5-200 scanning electron microscope. SEM images were taken of the upper leaf surface at a magnification of x3.5k

(Figure 3.15). Leaves from 4 plants per genotype or treatment were examined and ten individual stomata per leaf were photographed. A representative image of each was selected.

For cross-section imaging, leaf sections were prepared via fixation in Karnovsky's fixative, lipid fixation with osmium tetroxide and dehydration via ethanol series. Sections were then infiltrated and embedded in araldite resin. Five hundred-nm cross-sections were cut using a microtome and stained with 1% toluidine blue. Images were taken using a light microscope at a magnification of x100. These cross-sections allow the cuticular ledge of the stomata (indicated in Figures 3.15 C and F) to be more easily viewed. There was a degree of variation observed in the light microscopy imaging within genotypes so a representative image has been selected, however, the degree of variation can be seen in Appendix B.

A raised cuticular ledge was seen in SEM imaging of WT stomata (Figure 3.15A) and this phenotype remained unaffected by cold-acclimation (Figure 3.15B). Light microscopy cross-section imaging of WT plants also showed the raised cuticular ledge observed in SEM imaging (Figure 3.15C). The stomatal pore is indicated with an arrow and the raised cuticular ledge can be seen on either side. In contrast, *sfr8* imaging via SEM and cross section showed the absent stomatal cuticular ledge observed in *scord6* mutants (Figure 3.15D and F). Again, acclimation of *sfr8* did not impact its phenotype (Figure 3.15E).

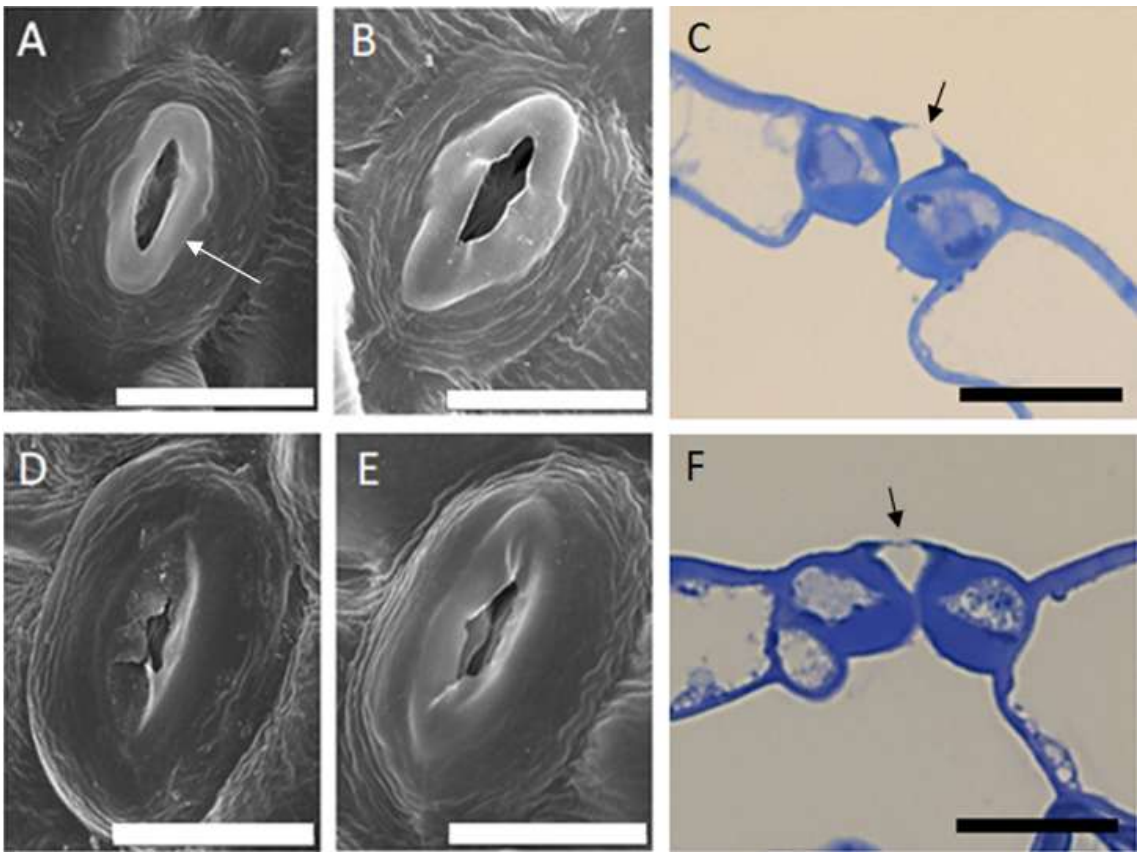

Figure 3.15: SEM images of the leaf surface and light microscopy cross-section images of stomata from 5-week-old WT and *sfr8* mutants.~40 SEM images of stomata were taken for each genotype and treatment and a representative image is shown. Images were taken at x 3.5k magnification and a scale bar of 10 µm can be seen on each image. **A)** Col-0 (WT) with white arrow indicating WT cuticular ledge, **B)** *sfr8*, **D)** acclimated WT, **E)** acclimated *sfr8*. Representative light microscopy images showing stomatal cross-sections in **C)** WT and **D)** *sfr8* plants. Images were taken at 100x magnification and a scale bar of 100 µm is shown. Black arrows indicate the stomatal pores and the cuticular ledge can be seen on either side.

The lack of cuticular ledge observed in *sfr8* was also seen in both *bor1-3/2-1* (Figures 3.16A and B) and *bor1-3* mutants (Figures 3.16C and D) in both the SEM images and cross-sections. In comparison, both *bor2-1* (Figures 3.16E and F) and *bor2-2* mutants (Figures 3.16E and F) show a WT-like phenotype and appear to have a raised cuticular ledge. *sfr8, bor1-3/2-1* and *bor1-3* all showed reduced dimerisation and display altered stomatal morphology while *bor2-1* and *bor2-2* showed both WT levels of RGII dimerisation and WT-like stomata. These results strongly indicate that defective RGII dimerisation may be linked to the altered stomatal morphology observed.

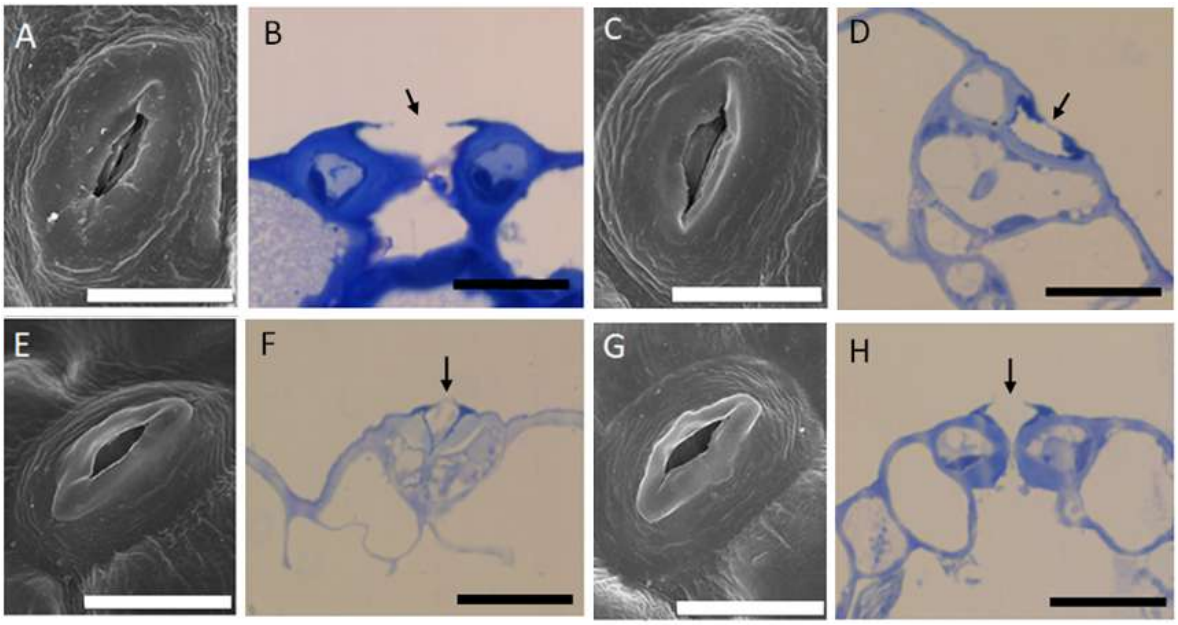

Figure 3.16: SEM images of the leaf surface and light microscopy cross-section images of stomata from 5-week-old *bor* mutants.~40 SEM images of stomata were taken for each genotype and a representative image is shown. Images were taken at x3.5k magnification and a scale bar of 10 µm can be seen on each image. 500 nm cross sections were taken for each genotype, stained with toluidine blue and imaged at 100x magnification on a light microscope. A representative image of each is shown with a 100-µm scale bar and arrow indicating the stomatal pore. SEM and light microscopy images are shown respectively for **A and B)** *bor1-3/2-1,* **C and D)** *bor1-3*, **E and F)** *bor2-1* and **G and H)** *bor2-2*. See Figure 3.15 for WT control.

3.2.5 The effect of BA supplementation of *bor1-3/2-1* stomatal morphology

Given the ability of supplementation with BA to restore both RGII dimerisation and freezing sensitivity in *bor1-3/2-1* leaves, the effect of supplementation on stomatal morphology was investigated. WT and *bor1-3/2-1* mutants both with and without BA supplementation were compared. It was shown that un-supplemented *bor1-3/2-1* displayed absent cuticular ledges (Figures 3.17 E and F) whereas supplemented *bor1-3/2-1* showed a WT-like phenotype (Figures 3.17G and H). Supplemented (Figures 3.17C and D) and un-supplemented WT (Figures 3.17 A and B) stomata were also compared and no change was seen. These results suggest that restoration of RGII dimerisation by BA supplementation can restore a WT-like stomatal phenotype. As time restraints did not allow BA supplementation to be investigated in all mutants *bor1-3/2-1* was selected for this experiment as RGII dimerisation was seen to be restored in this mutant.

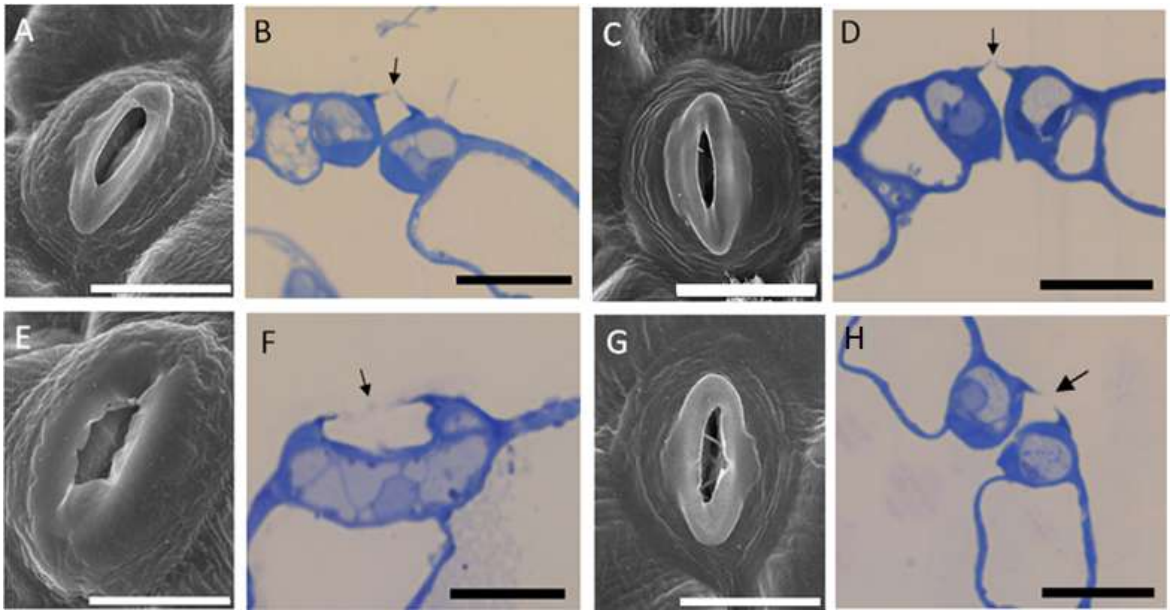

Figure 3.17: SEM images of the leaf surface and light microscopy cross-section images of stomata from 5-week-old WT and *bor1-3/2-1* with am without BA supplementation. ~40 SEM images of stomata were taken for each genotype and a representative image is shown. Images were taken at x 3.5k magnification and a scale bar of 10 µm can be seen on each image. 500 nm cross sections were taken for each genotype, stained with toluidine blue and imaged at 100x magnification on a light microscope. A representative image of each is shown with a 100-µm scale bar and arrow indicating the stomatal pore. SEM and light microscopy images are shown respectively for **A and B)** Col-0 (WT) **C and D)** WT + BA, **E and F)** *bor1-3/2-1* and **G and H)** *bor1-3/2-1* +BA.

The *sfr8, bor1-3* and *bor1-3/2-1* mutants all show defective RGII dimerisation and also show absent cuticular ledges and altered stomatal morphology. In contrast *bor2-1* and *bor2-2* both showed WT levels of RGII dimerisation and WT-like cuticular ledges. This indicates that

stomatal morphology is affected by RGII dimerisation levels in the leaves. It was also found that the stomatal morphology of *bor1-3/2-1* was restored when supplemented with BA. BA supplementation has also been shown to restore RGII dimerisation levels (O'Neill *et al.*, 2001), further indicating a link between RGII dimerisation and stomatal morphology.

3.2.6 TEM cross-section imaging of RGII dimerisation mutants

TEM imaging of WT, *sfr8* and *bor1-3/2-1* stomata were carried out to confirm the results seen in the cross sections viewed via light microscopy. 100 nm cross sections were taken from the samples prepared for light microscopy and TEM imaging was carried out by the Durham University electron microscopy department. The cuticular ledge of each stoma, highlighted using arrows, showed WT (Figure 3.18A) stomata had raised cuticular ledges while *sfr8* and *bor1-3/2-1* (Figure 3.18 B and C respectively) both showed absent cuticular ledges. These results concurred with the results seen via light microscopy for WT (Figures 3.15 C and 3.17 B), *sfr8* (Figure 3.15F) and *bor1-3/2-1* (Figures 3.16 B and 3.17 F) and therefore confirmed the accuracy of these results.

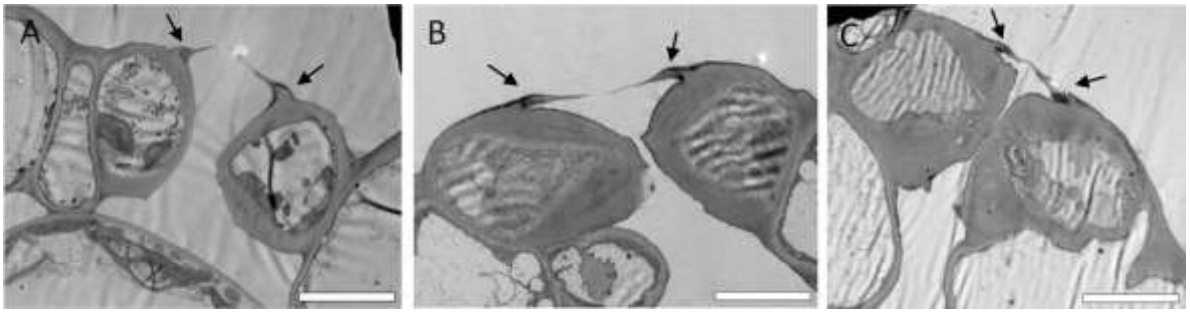

Figure 3.18: TEM cross section images of WT, *sfr8* and *bor1-3/2-1* stomata. 100 nm cross sections were taken from Col-0 (WT), *sfr8* and *bor1-3/2-1* leaves and imaged at 1200x magnification, a representative image is shown. Arrows indicate the cuticular ledge and a scale bar of 5 µm is show. **A)** WT, **B)** *sfr8* and **C)** *bor1-3/2-1*.

3.2.7 Stomatal density in RGII dimerisation mutants

Stomatal density was used instead of stomatal index given the time limitations as the epidermal cells proved very difficult and time consuming to count. While this method does not provide so much information about the leaf as a whole it is still important as the density per unit area can be seen. SEM images were taken at 400x magnification to investigate stomatal density in RGII dimerisation mutants. Images show an area of approximately 330 µm by 230 µm. Twelve images were taken per genotype and treatment, the stomata were counted and an average density per cm^2 was calculated for each (Table 3.1).

The effect of acclimation of WT and *sfr8* stomatal densities was investigated and it was observed that while densities were very similar in both acclimated and non-acclimated plants,

the density decreased for both genotypes upon acclimation. This may be due to the additional 2-week growth period allowing expansion of the cells resulting in more dispersed stomata.

The *bor2-1* and *bor2-2* mutants showed density very similar to that observed in WT leaves. In comparison *bor1-3* had approximately twice as many stomata per cm^2 than WT and *bor1-3/2-1* had an average three times as many stomata as WT per cm^2. When supplemented with BA the stomatal density per cm^2 of *bor1-3/2-1* reduced by more than 50% and approached WT-like levels. In comparison WT leaves appeared relatively unaffected by BA supplementation.

Genotype	Supplementation (+/-)	Acclimation (A/NA)	Density per cm^2	Standard Deviation
WT	-	NA	2.46	0.46
sfr8	-	NA	2.48	0.64
WT	-	A	1.85	0.47
sfr8	-	A	1.85	0.59
WT	-	NA	3.12	0.80
bor2-1	-	NA	3.10	0.90
bor2-2	-	NA	3.52	0.94
bor1-3	-	NA	6.08	0.76
bor1-3/2-1	-	NA	9.10	1.06
WT	-	NA	2.05	0.71
WT	+	NA	2.24	0.56
bor1-3/2-1	-	NA	7.63	2.63
bor1-3/2-1	+	NA	2.94	0.94

Table 3.1: Stomatal density of WT, *sfr8* and *bor* mutant leaves with various treatments. Twelve SEM images at 400x magnification were taken per genotype and treatment and the average stomatal density for each genotype per cm^2 was calculated.

The increase in stomatal density per cm^2 observed in *bor1-3* and *bor1-3/2-1* mutants could contribute to the increased rate of water loss observed in these mutants. This is supported by the ability of BA supplementation to restore both stomatal density (Table 3.1) and water loss (Figure 3.13) to WT-like levels.

Representative images showing the stomatal density observed in WT, *sfr8* and *bor1-3/2-1* leaves can be seen in Figure 3.19 A, B and C respectively. While WT and *sfr8* appeared to have similar stomatal density, *bor1-3/2-1* had much higher density. It also appears that *bor1-3/2-1* may have a cell expansion phenotype affecting the leaf cells. This can be seen in Figure 3.19C as the cells of the epidermis appear less expanded (smaller) causing the stomata to appear more tightly packed. As such, stomatal index may be a more reliable measurement to use for future experiments as it considers the ratio of epidermal cells to stomata.

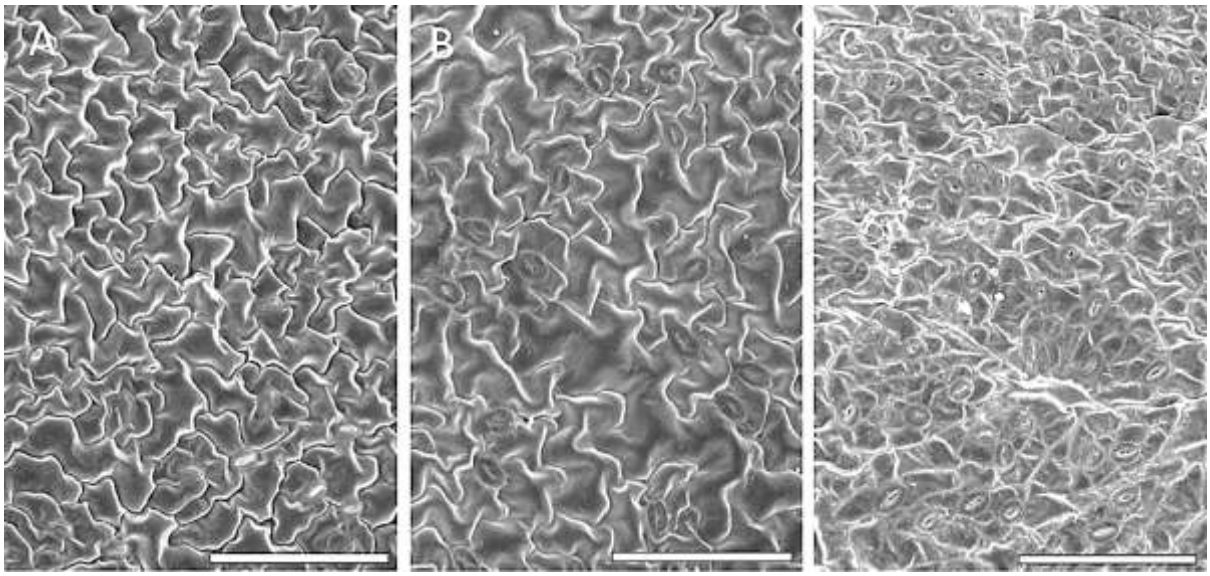

Figure 3.19: SEM images of WT, *sfr8* and *bor1-3/2-1* leaf surfaces. 12 images were taken per genotype and a representative image of each is shown. Images were taken at 400x magnification and a 100 µm scale bar is shown. **A)** Col-0 (WT), **B)** *sfr8* and **C)** *bor1-3/2-1*.

It is possible this cell expansion phenotype is linked to a decreased level of boron in *bor1-3/2-1* leaves caused by the lack of boron transporter proteins, but it is unlikely to be caused by a lack of RGII dimerisation as cell expansion is not altered in *sfr8* leaves.

CHAPTER 4

DISCUSSION

4.1 *sfr8* shows increased freezing sensitivity both with and without cold-acclimation

Freezing tolerance is essential for plants to survive sub-zero temperatures. This tolerance can be acquired through multiple pathways and several *SFR* (sensitive to freezing) genes have been identified, including freezing sensitive mutant *sfr8* (Warren *et al.*, 1996; Thorlby *et al.*, 1999). The cause of this freezing sensitivity was investigated by Panter (2019) using allelic freezing sensitive mutants *sfr8* and *mur1*. *sfr8* is a *MUR1* mutant, which is defective in its ability to produce fucose leading to decreased levels of RGII dimerisation. It is possible that fucosylation increases in response to cold-acclimation and that the lack of fucosylation in *sfr8* is responsible for the freezing sensitivity observed. However, it is also possible that the freeing sensitivity is caused by the lack of dimerisation of the cell wall pectin domain RGII which may also be triggered by cold-acclimation.

Cell wall modification and synthesis genes are highly represented in cold acclimation induced gene up regulation (Le, Pagter and Hincha, 2015). Acclimation may trigger many changes to the cell wall that increase freezing tolerance, such as altered cell wall content (Domon *et al.*, 2013) and increased thickness (Weiser, Wallner and Waddell, 1990) potentially leading to increased mechanical strength and cell wall rigidity as a protection mechanism against deformation by ice (Rajashekar and Lafta, 1996b; Solecka, Zebrowski and Kacperska, 2008). Arabionose side chains in RGI are thought to be involved in desiccation-tolerance (Moore, Farrant and Driouich, 2008). In addition, cold acclimation triggers increase in the levels of arabinose side chains present in RGI (Baldwin *et al.*, 2014). This is thought to prevent excess crosslinking and stabilize the cell wall during dehydration stress caused by freezing. Demethylation of cell wall pectin homogalacturonan (HG) is also triggered enabling crosslinking which is thought to influence both cell wall pore size and cell wall strength, both thought to be involved in freezing tolerance (Takahashi *et al.*, 2019).

Given the vast array of changes that occur as a result of cold acclimation it is possible that RGII dimerisation is also triggered by cold-acclimation. If so, the freezing sensitive nature of *mur1* mutants may be caused by a lack of cold induced RGII dimerisation. However, *sfr8* was identified in a screen for cold-acclimation mutants, therefore, previous research focused largely on acclimated plants and did not consider those which had not been acclimated (Thorlby *et al.*, 1999; Panter, 2019). As these studies focused on acclimated plants it remains

unclear if the defective RGII dimerisation, and potentially freezing sensitivity of the mutants, could instead be caused by an inherent defect rather than an inability to acclimate.

To determine the effect of acclimation on freezing tolerance acclimated and non-acclimated WT and *sfr8* plants were both qualitatively and quantitatively compared (Figures 3.1, 3.2 and 3.3). When quantitatively examined by EL assay *sfr8* was found to be significantly more freezing sensitive than WT both with and without a period of acclimation but showed improved freezing tolerance after acclimation (Figure 3.3). This indicated that *sfr8* was not defective in its ability to acclimate and that the abilities to sense and respond to external cold stimuli were not compromised.

Qualitative plate freezing assays (Huang *et al.*, 2017) were explored as a method for investigating freezing tolerance using seedlings. It was thought this method may prove a useful alternative to EL assays for several reasons: the use of seedlings reduces the plant growth time to less than half required for an EL assay, the number of genotypes or treatments tested per experiment could be increased and the assay itself is less labour intensive and time consuming. However, as described in the results the watery consistency of ½ x MS agar post freezing presented an issue as the shortened petioles of *sfr8* seedlings (Reiter, Chapple and Somerville, 1993; Panter, 2019) increased the chance of anoxia in these seedlings. This could, therefore, have led to inaccurate results with higher levels of damage to the mutants than would be seen with freezing alone. To address this issue increasing the agar content of the media to increase the strength was considered however, when agar is too hard it prevents the growth of the roots into the media (Roué *et al.*, 2019). While seedlings can still grow like this, roots are not adapted to withstand freezing as they are normally protected by the soil therefore, seedlings with exposed roots would not be suitable for this assay as exposed roots could lead to an overrepresentation of the level of damage freezing causes. As an alternative 2% sucrose was added to the media as shown by Huang *et al.* (2017), this also prevents the media 'splitting' and forming a watery layer. However, this presented issues of its own; when seedlings were allowed to recover at 20°C plates were very quickly colonised by yeast preventing clear observation of the results. To combat this, plants were allowed to recover at 5°C but the decreased temperature inhibited any further growth of seedlings that had survived, making it hard to see differences. In addition, the high stress caused by the cold led to a build-up of anthocyanin (Christie, Alfenito and Walbot, 1994) causing the purple leaves seen in Figure 3.2. While this method also showed higher freezing sensitivity in *sfr8* seedlings than WT and an increase in freezing tolerance in both WT and *sfr8* upon acclimation, the technical issues with this experiment meant EL remained a more reliable assay.

Although *sfr8* was identified in a mutant screen (Warren *et al.*, 1996) for cold-acclimation mutants this was carried out more than twenty years ago. *sfr8* may have been mistakenly identified as a cold-acclimation mutant in this screen because acclimated *sfr8* shows reduced freezing tolerance when compared to acclimated WT. Likewise, a comparison of *sfr8* freezing sensitivity before and after acclimation was never done or the improved freezing sensitivity of *sfr8* post acclimation would have been seen. Had these results been observed they would have indicated that while *sfr8* is a freezing sensitive mutant it is not a cold-acclimation mutant. While cold-acclimation has been more extensively studied some innately freezing tolerant mutants have previously been identified (Xin and Browse, 1998).

In addition, Panter *et al.* (2019) showed transcription factor *CBF1-3,* involved in cold-regulated gene expression (Gilmour, Fowler and Thomashow, 2004), was expressed at WT levels in *sfr8*. Several CBF-controlled *COR* genes were also shown to have WT-like levels of expression indicating that freezing sensitivity observed in *sfr8* is not caused by interruption to CBF controlled pathways (Panter *et al.* 2019). Therefore, it can be concluded that *sfr8's* freezing sensitivity is not caused by defective acclimation and may instead be the result of inherent freezing sensitivity. This leads to the question of what role *MUR1* has in cold acclimation and freezing sensitivity.

4.2 *sfr8* is freezing sensitive and has defective RGII dimerisation

Both *mur1-1* and *sfr8* are compromised in their ability to synthesis L-fucose and therefore in their ability to form dimeric RGII. This was seen in cell wall AIR extracts of the mutants which showed monomeric RGII and therefore a decrease in dimeric RGII in comparison to WT. Supplementation with fucose was able to restore dimerisation to WT-like levels in both *sfr8* and *mur1-1* (Figure 3.4). This was also seen by Panter (2019) and, in addition, fucose supplementation was also shown to restore freezing tolerance to WT-like levels in *sfr8*.

Restoration of RGII dimerisation, as shown by O'Neill *et al.* (2001) and seen in Figure 3.4, could be responsible for the restored freezing tolerance observed when *sfr8* is supplemented with fucose. However, fucosylation is important for many aspects of the cell wall. *mur1* mutants also show defective fucosylation of arabinogalactan proteins (APGs) (Tryfona *et al.*, 2012) and Xyloglucans (Rayon *et al.*, 1999; Perrin *et al.*, 2003). To determine if the freezing sensitivity observed in *mur1* and *sfr8* mutants is caused by defective RGII dimerisation and not a product of the lack of fucosylation, other mutants that were also compromised in their ability to dimerise RGII were investigated.

4.3 RGII dimerisation mutants show freezing sensitivity

bor2 mutants appeared WT-like in phenotype whereas a stunted leaf phenotype was observed in *bor1-3* which was seen to an even greater degree in *bor1-3/2-1*. To ensure these mutants were suitable for freezing sensitivity experiments the levels of RGII dimerisation in the *bor* mutant leaves were investigated as freezing sensitivity is measured on leaves. Leaf cell wall AIR extracts were examined via gel electrophoresis and *bor1-3* and *bor1-3/2-1* both showed monomeric RGII, and therefore a decrease in the proportion of dimeric RGII. However, *bor2-1* and *bor2-2* displayed no monomeric RGII and showed WT-like dimerisation (Figure 3.5). This suggests that *BOR1* is required for RGII dimerisation in leaves but *BOR2* may not be.

It is likely that this is a result of the different roles the proteins play in boron transport. BOR1 is an efflux boron transporter required for xylem loading (Takano, Miwa and Fujiwara, 2008) while BOR2 is thought to be involved in transport of boron into the apoplast (Miwa *et al.*, 2013). *bor2* mutants showed a reduced root growth when compared to *bor1-3* and *bor1-3/2-1* and in contrast *bor1-3 and bor1-3/2-1* showed reduced shoot growth in comparison to *bor2* mutants. This suggests that the BOR1 protein is more involved in transporting boron to the aerial parts of the plant and could explain why mutants lacking functional BOR1 show decreased RGII dimerisation in the leaves. In comparison the BOR2 protein appears to be more important for root elongation, suggesting its main role is transport of boron within the roots. This could explain why the *BOR2* mutants did not show a decrease in leaf RGII dimerisation.

The reduced RGII dimerisation of *bor1-3* and *bor1-3/2-1* mutants confirmed their suitability for the experiment so an EL assay was carried out on the *bor* mutants to investigate their freezing sensitivity. Both *bor1-3* and *bor1-3/2-1* showed significantly higher levels of leakage, and therefore damage, when frozen than WT. In comparison *bor2-1* and *bor2-2* both showed leakage levels very similar to those seen in WT leaves (Figure 3.8 and appendix A). This indicated that *bor1-3* and *bor1-3/2-1* were significantly more freezing sensitive than both WT and *bor2* mutants. When freezing sensitivity and RGII dimerisation levels are compared it can be seen that the mutants that displayed freezing sensitivity, *bor1-3* and *bor1-3/2-1*, also showed defective RGII dimerisation. Similarly, the *bor2* mutants displayed WT-like freezing sensitivity and also had WT-like RGII dimerisation. This indicates that RGII dimerisation levels may be linked to freezing sensitivity.

bor1-3 and *bor1-3/2-1* are defective in their ability to dimerise RGII via a very different mechanism than that seen in *sfr8*. The *bor* mutants show decreased dimerisation as a result of inhibited boron transportation through the plant while the lack of L-fucose synthesis in *sfr8* is responsible for the defective RGII dimerisation observed there. As the *bor* mutants are not

affected in their ability to synthesise fucose the sensitivity to freezing observed in these mutants cannot be attributed to a lack of fucosylation of cell wall components, as suspected in *sfr8*. Therefore, as the consistent defect between *bor1-3, bor1-3/2-1* and *sfr8* is their inhibited ability to dimerise RGII this is extremely likely to be responsible for the freezing sensitivity observed.

sfr8 and *bor* mutants both show defective RGII dimerisation without a period of acclimation, therefore, unlike the vast array of cell wall changes controlled by cold-acclimation RGII dimerisation appears to be controlled independently of the cold-acclimation process despite being linked to freezing tolerance. This suggests there may be a degree of innate freezing tolerance in WT plants and the lack of this in *sfr8* and *bor* plants suggests that RGII dimerisation is involved. Innate freezing tolerance has been observed in *esk1* mutants by Xin and Browse (1998). This mutant showed WT-like expression of *COR* genes, known to be upregulated during cold-acclimation, strongly suggesting that the freezing tolerance observed was independent of cold-acclimation. The results seen in *sfr8* and *bor* mutants indicate that RGII dimerisation many contribute to innate freezing tolerance in plants. It was also shown in cell culture that RGII monomer formed in the absence of boron cannot subsequently form dimeric RGII upon supplementation with BA. This indicates that dimerisation either occurs in the protoplasm prior to secretion into the cell wall or during the secretion process (Chormova, Messenger and Fry, 2014). Therefore, the majority of dimerisation is likely to occur early in the growth period, again indicating acclimation is not involved in RGII dimerisation and that it is likely to be an innate form of freezing sensitivity.

4.4 Boron supplementation restores both RGII dimerisation and freezing tolerance in mutants

Supplementation with boric acid (BA) was expected to restore dimerisation as it restores other phenotypes caused by a lack of dimerisation, such as the growth phenotype (Ishii, Matsunaga and Hayashi, 2001; O'Neill *et al.*, 2001; Miwa *et al.*, 2013) and *sfr8* freezing sensitivity (Panter et al 2019). It has also been seen to restore RGII crosslinking to WT levels in *mur1* mutants (O'Neill *et al.*, 2001; Voxeur *et al.*, 2011), however, this was not seen by Panter *et al.* (2019). Supplementation with BA does not restore L-fucose synthesis and RGII side chain A remains truncated (O'Neill *et al.*, 2001). This is believed to cause instability in the dimerised form of RGII, leading to the decrease in RGII dimerisation observed in *mur1* mutants (O'Neill *et al.*, 2001; Sechet *et al.*, 2018). The high concentration of boron created by supplementation makes the dimerisation interaction more likely however, the truncated side chains lead to weaker inter-molecular bonds and unstable RGII dimerisation (O'Neill *et al.*, 2001; Sechet *et al.*, 2018). It is thought that this instability means the dimer cannot withstand AIR extraction from

the cell wall and the procedure leading up to gel electrophoresis and, therefore, restored dimerisation will not be observed. Support for this was given by Begum and Fry (personal communication) who observed that restored RGII dimer was not able to survive the AIR preparation procedure as the process caused it to return to the monomeric state. Previous experiments that have shown restoration of RGII dimerisation used alternative methods to quantify dimerisation levels such as size exclusion chromatography (Zhong *et al.*, 2005) and gel filtration chromatography (Albersheim *et al.*, 2011). These methods do not damage the fragile double bonds allowing restored RGII dimerisation to be observed. This supports the explanation that even if dimerisation was restored in the *sfr8* mutants tested by Panter (2019) the fragile bonds would make it impossible for RGII dimerisation to be visualised via gel electrophoresis. Therefore, it remains unclear if dimerisation was restored but subsequently lost due to the fragile nature of the dimer or if dimerisation was simply never restored.

The ability of BA supplementation to restore freezing sensitivity in *sfr8* plants was a good indication that RGII dimerisation is involved in freezing tolerance. However, the lack of proof that RGII dimerisation levels were also restored by BA meant further clarification was required. Cost restraints prevented the use of size exclusion or gel filtration chromatography to confirm RGII dimerisation levels in *sfr8*. However, as discussed, BA supplementation has been shown to restore RGII dimerisation levels in the roots of *bor2* mutants (Miwa *et al.*, 2013). Similarly, BA supplementation restored WT leaf expansion and fertility in previous studies on *bor1* mutants (Noguchi *et al.*, 1997; Takano *et al.*, 2002). Leaves from supplemented and un-supplemented *bor* mutants, also defective in their RGII dimerisation, were investigated to further clarify the relationship between RGII dimerisation and freezing sensitivity. Initially it was seen that supplementation with BA was able to restore the stunted leaf phenotype of *bor1-3/2-1* mutants to WT-like (Figure 3.10). Supplementation with BA was also able to restore freezing tolerance to levels similar to those seen in WT plants confirming that supplementation restores freezing tolerance in RGII dimerisation mutants (Figure 3.11). As these mutants do not have the truncated side chain present in *sfr8* mutants, the restored dimerisation was visible via gel electrophoresis as the dimer was not unstable. Dimerisation was restored to WT-levels in *bor1-3/2-1* upon supplementation with BA (Figure 3.10). Together these results and the results from the *sfr8* mutant plants further implicate RGII dimerisation in freezing tolerance.

4.5 Boron transport

The lack of BOR1 transporter in *bor1-3* and *bor1-3/2-1* caused the reduced RGII dimerisation seen in leaves (Figure 3.5). However, supplementation was seen to be effective in restoring RGII dimerisation despite the lack of transporter proteins (Figure 3.10). This shows that 'flooding' the plant with excess boron enables uptake of boron at high enough levels to restore

dimerisation. Therefore, plants must be able to use alternative methods to transport BA. As previously discussed BOR1 is required for transporting boron against the concentration gradient into the xylem (Takano, Miwa and Fujiwara, 2008). Therefore, even if cellular transporters were present in the leaves little or no boron could reach the aerial tissue and limited dimerisation would occur. This explains why mutants without BOR1 transporters, *bor1-3* and *bor1-3/2-1*, show decreased RGII dimerisation in the leaves (Figure3.5) as boron transport is disrupted (Takano *et al.*, 2005; Takano, Miwa and Fujiwara, 2008; Miwa *et al.*, 2010).

In comparison BOR2 is thought to be required for transport of boron from the symplast into the apoplast (Miwa *et al.*, 2013). Therefore, it is expected that it would be present in all cells and *bor2* mutants would show defective RGII dimerisation. However, decreased dimerisation is observed in *bor2* roots (Miwa *et al.*, 2013) but not in leaves. The reason for this remains unexplained as *BOR2* has been shown to be expressed in the epidermal cells of leaves (Takada *et al.*, 2014). An explanation could be that while BOR2 proteins exists in leaves, they do not play an essential role in the transport of boron in leaf cells. It is possible that if high enough levels of boron reach the leaves transport can be carried out via alternative pathways. This would explain why *bor2* single mutants can show normal RGII dimerisation and freezing tolerance levels in the leaves (Figures 3.5, 3.6 and 3.8). In contrast, *bor1* mutants show very low levels of RGII dimerisation in leaves due to inhibition of boron transport into the xylem. This explanation could also account for the fact *bor1-3/2-1* mutants show higher levels of freezing sensitivity than *bor1-3*. As *bor2-1* mutants do not have altered freezing sensitivity it could be expected than a lack of BOR1 is causing the freezing sensitivity and that therefore the double mutant would show similar damage to that observed in *bor1-3*. However, *bor1-3/2-1* damage is much more severe suggesting BOR2 may have a role in boron transport in leaves under boron limiting conditions. This is supported by data showing *bor1-3/2-1* supplementation with a GFP-BOR1 construct was able to almost fully restore the growth phenotype to WT-like (Kasai *et al.*, 2011). This suggests that when the levels of boron in the leaves are severely compromised by a lack of boron transport in both roots and xylem loading the role BOR2 plays in leaves becomes much more important. This would explain the more sever phenotype observed in the *bor1-3/2-1* mutant.

While essential for plant growth and development, boron is toxic to plants in high quantities (Goldberg, 1997). Various methods of supplementation can be used such as seedling growth on agar (Voxeur *et al.*, 2011; Villalobos, Yi and Wallace, 2015), spraying (O'Neill *et al.*, 2004; Zhong *et al.*, 2005) or hydroponics (Miwa *et al.*, 2013). The concentration of BA used in these methods varied from 90 mg/L to 5 g/L as a result of the differences in delivery method,

frequency of delivery and the age of the plants. When grown on soil plants were supplemented with 20 mg/L BA as this was the level shown to restore WT freezing tolerance and appearance in *sfr8* plants (Reiter, Chapple and Somerville, 1993; Panter, 2019). However, WT plants sometimes showed yellowing of the leaves, which may be an indication of boron toxicity. Toxicity in WT plants may be the cause of some of the varying results observed when supplementing plants with BA. While freezing sensitivity and leaf shape of WT plants were not seen to be affected by BA supplementation (Figure 3.11), variation was observed in the desiccation rates of supplemented WT plants (Figures 3.12 and 3.13). This indicated that BA supplementation may have been influencing the rate of water loss from the leaves in WT plants as BA was clearly not without some effect on WT plants.

Investigating the hydroponic growth system showed supplementation with as little as ¼ x MS growth medium, which contains 1.55 mg/L BA, was sufficient for full phenotypic restoration of *bor1-3/2-1* to a WT-like phenotype (Figure 3.9). It was assumed that the lower levels of BA required for supplementation were due to efficient uptake in this growth system as opposed to plants grown on soil. However, it may be that the mutant phenotype in *bor* plants is more readily restored than in *sfr8* plants. This is indicated by Miwa *et al.* (2013) where leaf expansion was shown to be restored with 30 µM BA and fertility with 100 µM. The low levels of BA required for supplementation in this system make it difficult to compare supplemented to un-supplemented plants as reducing MS levels low enough to limit boron availability to the extent necessary to reveal the typical mutant phenotype would also reduce the levels of other nutrients required for plant growth and development, compromising survival. This could be addressed by supplementing plants with Hoaglands solution (Hoagland and Arnon, 1938) rather than MS. This would allow individual components to be adjusted as needed and boron could be added in very low doses or removed entirely without depriving the plants of other essential nutrients. However, given both equipment and time restraints, large scale hydroponic growth systems were not pursued. Further investigation into BA supplementation on soil grown plants could be used to establish a more precise concentration of BA required for supplementation and may help determine if BA toxicity is impacting results.

WT plants show normal growth on peat plugs without supplementation. This suggests there is either enough boron in the soil naturally or the requirement for boron occurs early in the plant's life cycle. This could happen if RGII dimerisation occurs early in the plants growth cycle as suggested by Fry (personal communication) and Miwa *et al.* (2013). If so, the plants' boron requirement for RGII dimerisation may be met during the initial stages of growth in which seedlings are germinated and grown for approximately 2-weeks on agar supplemented with MS and containing BA. Further investigation into boron requirements at different stages of the

growth cycle could also help address the issue of boron toxicity without compromising growth or development.

4.6 The effect of stunted leaf phenotype in *bor* mutants

The decreased volume to surface area ratio observed in the stunted leaves of both *bor1-3* and *bor1-3/2-1* mutants presented issues for both EL and leaf drying assays. Comparison of WT leaves with *bor* mutant leaves was difficult as matching for age and developmental stage caused a large degree of variation in leaf size and matching for size resulted in comparison between very young leaves and much older and more developed leaves. As neither matching leaves for age nor size allowed for a straightforward comparison both methods were used and the results compared. The difficulties faced in comparing WT and *bor* mutant leaves are explored further below.

4.6.1 Effect of leaf size on freezing sensitivity

Previous EL experiments used size-matched leaves (Hemsley *et al.*, 2014) as this method removes any variation in surface area to volume ratio between the mutants. However, due to the small size of the *bor1* mutant leaves very young WT leaves had to be used to match for size. In this experiment (Figure 3.6A), young WT and *bor2-1* leaves were compared to older *bor1-3* and *bor1-3/2-1* leaves of the same size. Significantly higher levels of leakage were seen in *bor1-3* and *bor1-3/2-1* when compared to WT and *bor1-2* indicating they were significantly more freezing sensitive. However, very young leaves are not fully developed (Van Lijsebettens and Clarke, 1998; Bar and Ori, 2014) and may be more sensitive to freezing that older leaves. Therefore, the damage seen to the small, underdeveloped WT leaves may not be representative of the damage caused by freezing. Thus, the difference between WT and *bor1-3* and *bor1-3/2-1* may be underrepresented in this experiment. Matching leaves for age and developmental stage removes the issue of underdeveloped leaves and showed very similar results (Figure 3.6B). However, in this case the size variation between the leaves was extreme with very small *bor1-3* and *bor1-3/2-1* leaves being compared to much larger WT leaves. This could result in the smaller leaves experiencing greater damage when frozen due to their decreased surface area to volume ratio and could therefore overrepresent the difference in freezing tolerance between WT and *bor1-3* and *bor1-3/2-1*. However, significant differences were observed between WT and *bor1-3* and *bor1-3/2-1* irrespective of whether leaves were matched for size or age. Given that matching leaves by size is more likely to lead to under-representation of the differences in leakage between groups, this was the method taken forward for further experiments using *bor* mutants.

To investigate the effect of size and age further, an EL assay was carried out comparing very small juvenile or young leaves to large, medium aged WT leaves (Figure 3.7). To allow a similar

amount of tissue to be examined for each size and age, either 3 small leaves or 1 large leaf was used. In retrospect this was a design flaw in the experiment as the use of single large leaves caused the variation in the results to be greatly increased. As a result of the large standard deviation in the large, medium aged leaves the results were not seen to be significantly different when using an ANOVA test. However, it can be seen that on average small leaves, whether young or juvenile, were more freezing-sensitive than large leaves. This would suggest that size is an important factor in EL assays, however, further investigation into this would need to be carried out before this could be said with certainty.

These experiments indicate that in leaves, both reduced size and potentially extremes in age, cause increased freezing sensitivity. As leaf size is a function of developmental sequence the first leaves produced by the plant are always small as are the most recently produced leaves that have not yet fully developed (Figure 3.7 A). As such, size and age are linked making it difficult to assess these factors independently from each other.

To address this difficulty and further investigate the effect of size and age on freezing sensitivity a preliminary experiment using other well-studied mutants with stunted phenotypes, was carried out. Gibberellin signalling mutant *sly1* (McGinnis *et al.*, 2003) and constitutive ethylene signalling mutant *ctr1* (Kieber *et al.*, 1993), which are defective in cell expansion and cell division, respectively, both have smaller leaves than WT plants. However, these mutants have never been shown to display a freezing sensitive phenotype. A provisional EL assay (not shown) comparing these mutants with WT plants indicated that size was inversely correlated with freezing tolerance. This experiment allowed the age and developmental stage of the leaf to remain the same while the size varies. The use of several size mutants which had not been seen to show freezing sensitivity meant the only difference between the WT and mutant leaves was size, suggesting any difference in freezing sensitivity could be attributed to differences in size. This would need to be repeated to confirm these results, but the initial experiment would suggest that smaller leaves are more susceptible to freezing. This suggests that using age-matched tissue may not have been reliable in the case of the *bor* mutants and supports the decision to use size matched leaves to avoid the possibility of small *bor1-3* and *bor1-3/2-1* leaves leading to an overrepresentation of their freezing sensitivity. However, this experiment also has some confounding factors. A dual mechanism of reduced cell division in early growth followed by inhibition of cell expansion is thought to be responsible for the stunted growth phenotype observed in *sly1* mutants. As such both low cell number and small cell size are responsible for the stunted phenotype of *sly1* mutants (Achard *et al.*, 2009). Similarly *ctr1* mutants have decreased cell size in comparison to WT (Kieber *et al.*,

1993). As such, it is possible that the decrease in cell size may be responsible for the increased freezing sensitivity observed in these mutants rather than the small leaf size.

4.7 Desiccation in RGII dimerisation mutants

Stomata are found on the leaves or stems of vascular plants and are composed of a guard cell pair and the pore between them (Heath, 1938; Willmer and Fricker, 1996; Hetherington and Woodward, 2003). The complex array of genes and mechanisms that control stomatal development and function are still being investigated today (Nadeau and Sack, 2002; Bergmann and Sack, 2007; Hunt *et al.*, 2017; Allen *et al.*, 2019). Functional stomata are extremely important for plant survival; they allow the plant to respond to environmental conditions as they regulate water loss from leaves via transpiration. Plants can alter the turgor of their guard cells by increasing osmotic water uptake to change their shape (Heath, 1938; Cowan and Troughton, 1971). This response is triggered by environmental stimulus such as light or drought or hormonal signals like abscisic acid (ABA) or auxin (Zeiger and Hepler, 1977; Lohse and Hedrich, 1992; Pei *et al.*, 1998; Schroeder *et al.*, 2001; Roelfsema and Hedrich, 2005). This alters the size and aperture of the stomata pore and allows the plant to regulate gas exchange. Guard cells are involved in the exchange of oxygen and carbon dioxide during photosynthesis and also allow water vapour to exit the leaves (Heath, 1938; Cowan and Troughton, 1971). While some water loss can occur through cell wall pores, the majority is lost via the stomata. Therefore, when experiencing drought conditions plants respond by decreasing the turgor of their guard cells, allowing the stomatal pore to close, thus conserving water (DeMichele and Sharpe, 1974; Franks *et al.*, 1995).

As plants respond to drought by closing their stomata to conserve water the plant's response to drought conditions can be tracked via water loss. A leaf drying experiment was carried out to investigate the stomatal response of plants to drought. Leaves were excised from the plant and weighed every hour for 8 h and then again at 48 h and 7 days. Removing the leaves triggers the drought response in the leaves causing the stomata to close and allows the initial phase of water loss to be observed (Lösch, 1979). The final measurement can be used to show the dry weight and the initial water content of the leaf.

It was seen by Panter (2019) and shown in Figure 3.12 that *sfr8* plants have a much faster initial rate of desiccation and reach almost complete dryness far quicker than WT. As mentioned, supplementation with BA may have been able to restore or partially restore WT-like desiccation rates, however, variation was seen in the results, suggesting boron toxicity may be occurring in the WT plants, as previously discussed. These results show that desiccation is altered in *sfr8* and suggest there may be altered stomatal behaviour or function

in these mutants. To investigate whether this could be linked to defective RGII dimerisation *bor* mutants were investigated.

When investigating the desiccation rates of *bor1-3/2-1* (Figure 3.13) leaf size also presented a problem as these mutants had a decreased surface area to volume ratio. While this has a large impact on water loss (Wang *et al.*, 2019), matching leaves for developmental stage was considered more important in this case to avoid compromise in stomatal development, density or reactivity by using leaves in early stages of growth and development (Jordan, Brown and Thomas, 1975; Nadeau and Sack, 2002; Miyazawa, Livingston and Turpin, 2006; Peel *et al.*, 2017). A faster initial rate of water loss was seen in *bor1-3/2-1* leaves allowing the maximum water loss to be reached much more quickly than WT (Figure 3.13). This may suggest that RGII dimerisation is involved in water loss via stomata. However, the difference in leaf size between the WT and mutant line may have impacted upon the rate of water loss in *bor1-3/2-1*. The small surface area to volume ratio of *bor1-3/2-1* could result in faster water loss (Wang *et al.*, 2019).

The effect of BA supplementation on *bor1-3/2-1* water loss was also investigated and showed that BA was able to restore WT-like leaf water loss rates. As BA is able to restore RGII dimerisation to WT levels (Figure 3.10) this could indicate that reduced RGII dimerisation in un-supplemented *sfr8* and *bor1-3/2-1* is responsible for the increased rates of water loss in these mutants. However, BA supplementation also restored the size and shape of *bor1-3/2-1* leaves to WT-like, which restored the surface area to volume ratio of the *bor1-3/2-1* plants to WT levels. Therefore, BA restoration of RGII dimerisation in *bor1-3/2-1* may not be involved in restoring WT-like water retention. Faster initial water loss in un-supplemented *bor1-3/2-1* mutants could be due to their small size and restoration of WT-like desiccation in BA supplemented *bor1-3/2-1* could be due to restoration of WT leaf size.

4.7.1 Effect of leaf size in desiccation experiments

An alternative approach to investigate desiccation is the use of thermal imaging (Jones, 1999; Merlot *et al.*, 2002; Wang *et al.*, 2004). This allows the effect of drought to be observed for a whole plant rather than an individual leaf and is a more accurate representation of the conditions a plant would encounter during a severe drought. The temperature of the leaf can be used as an indicator of the level of transpiration that is occurring on the leaf surface. When plants are transpiring, their leaves are cooler as evaporative cooling is occurring on the leaf surface (Merlot *et al.*, 2002). In response to drought plants usually shut their stomata and stop transpiring to conserve water. Thermal imaging was used to further investigate desiccation in RGII dimerisation mutants using WT and *bor1-3/2-1* plants. Plants were kept at 100% humidity for 24 h prior to the experiment to ensure the highest level of saturation across the genotypes.

Plants were then placed at ambient room temperature and imaged every 30 mins for 4 days allowing the plants' response to drought conditions to be observed (Figure 3.14). *bor1-3/2-1* plants were initially cooler than WT plants, indicating that higher levels of evaporative cooling were occurring or that the stomata were more open. After approximately 24 h *bor1-3/2-1* plants remained cooler than WT plants however, both genotypes were seen to have increased their temperature by a similar degree. This suggested that the both WT and mutant plants were able to respond to the drought conditions by closing their stomata to reduce water loss. As *bor1-3/2-1* plants were able to respond to the drought conditions in a similar manner to that seen in WT, their ability to respond to external stimuli did not appear to be compromised. However, *bor1-3/2-1* plants still remained cooler than WT, suggesting they were still undergoing a higher level of evaporative cooling which continued for the remainder of the experiment. The increased water loss observed in *bor1-3/2-1* mutants is likely to be caused by having either a higher density of stomata (Eisenach *et al.*, 2017; Bertolino, Caine and Gray, 2019) or stomata that remain more open (Liang *et al.*, 2005; Li *et al.*, 2008; Daszkowska-Golec *et al.*, 2013).

While thermal imaging of whole plants may be a more reliable indicator of desiccation rates, the difference in size between WT and *bor1-3/2-1* plants remains an issue. Both *bor1-3/2-1* and WT plants were grown on the same sized peat plugs, however *bor1-3/2-1* plants were smaller than WT, therefore *bor1-3/2-1* plants are likely to have been using the available water more slowly and not experiencing the drought conditions to the same degree as WT plants. This could explain the increased cooling observed in *bor1-3/2-1* plants as they may not need to preserve water in the same way larger WT plants do. To compare stomatal responses more accurately and account for the rate that water is being taken up, the soil moisture content could be measured over time to ensure all plants are experiencing the same drought conditions (Susha Lekshmi, Singh and Shojaei Baghini, 2014).

Given the challenges with the decreased leaf size of the *bor* mutants it may be easier to investigate stomatal function independently of drought. One potential method would be to measure stomatal conductance in response to changing CO_2 levels. This takes the rate of CO_2 entering or water vapour exiting the cell as an indication of stomatal behaviour, density and aperture (Mcelwain, Yiotis and Lawson, 2016). In addition, accounting for leaf area could provide a more accurate indication of stomatal function in RGII dimerisation mutants. This information could indicate whether the diminished response to drought observed in desiccation experiments is an artefact of the small leaf size of *bor1-3/2-1* or if it is caused by altered stomatal behaviour or morphology. Given the reasons discussed above, desiccation

experiments cannot determine whether RGII dimerisation is responsible for controlling leaf water loss via the stomata.

4.8 Stomatal morphology in RGII dimerisation mutants

The altered desiccation patterns observed in *sfr8* and *bor1-3/2-1* (Figures 3.12 and 3.13) might indicate that RGII dimerisation has an effect on stomata. Desiccation experiments suggest that *bor1-3/2-1* is able to respond to environmental stimuli. However, these mutants appear to respond to a lesser extent suggesting stomatal density, morphology or closing behaviour may be affected.

An unusual stomatal morphology was identified in *MUR1* mutant *scord6* by Zhang *et al.* (2011). The raised ridge or cuticular ledge that normally forms around the stomatal pore was missing in *scord6* and *mur1-1* mutants. These mutants also displayed reduced stomatal closure in response to pathogens (Zhang *et al.*, 2019). This could suggest that the cuticular ledge is important for stomatal closing behaviour and, as *sfr8* is also a *MUR1* mutant, may explain the diminished drought response observed during the desiccation experiments. *sfr8* stomata were examined using both scanning and transmission electron microscopy to determine if the absent cuticular ledge observed in other *MUR1* mutants was present. The *sfr8* stomata showed a similar phenotype to that reported for *scord6,* both with and without a period of cold-acclimation (Figure 3.15 and 3.18).

To investigate whether this could be linked to RGII dimerisation *bor* mutants were investigated: *bor1-3* and *bor1-3/2-1*, shown to have altered RGII dimerisation, displayed the altered stomatal ridge phenotype. In comparison both *bor2* mutants, which do not have defective leaf RGII dimerisation, displayed WT stomata (Figure 3.16 and 3.18). All mutants defective in RGII dimerisation displayed altered stomatal morphology indicating that RGII dimerisation is involved in the formation of the cuticular ledge. Interestingly, when *bor1-3/2-1* was supplemented with BA, and RGII dimerisation was restored, restoration of the cuticular ledge was also observed (Figure 3.17).

These results would suggest that defective RGII dimerisation is the cause of the altered stomatal morphology observed as mutants with defective RGII dimerisation showed the same phenotypic alteration. In addition, the altered morphology was resolved when dimerisation levels were restored to WT via BA supplementation of *bor1-3/2-1*. To fully resolve this, the effect of BA supplementation on *sfr8* stomatal morphology could be investigated. However, this investigation was not carried out due to time constraints. In addition, the altered stomatal response to pathogens observed by Zang *et al*. (2011) may suggest the cuticular ledge is

required for stomatal closure which could explain the altered drought response observed in *bor1-3/2-1*.

4.8.1 Stomatal morphology and desiccation

The absent stomatal ledge phenotype observed in both *sfr8* and *bor1-3/2-1* may be responsible for the increased rate of water loss observed in these mutants. This alteration to the cuticular ridge may impact the ability of the stomata to open and close correctly, therefore resulting in a faster rate of desiccation. Other mutants have been identified as having either over and under developed cuticular ledges leading to decreased and increased transpiration rates respectively (Li *et al.*, 2007; Hunt *et al.*, 2017). This led to the hypothesis that the cuticular ledge may be affecting the ability of the stomata to open and close which could explain the altered desiccation rates observed in mutants with defective cuticular ledges including the RGII dimerisation mutants.

To investigate the functionality of stomata with altered morphology ABA and light could be used to trigger stomatal closure and opening respectively (Schroeder, Kwak and Allen, 2001; Shimazaki *et al.*, 2007). Observing the response to these stimuli under the microscope could be used to determine whether the mutants are affected in their ability to respond to external stimuli. The thermal imaging results from the *bor1-3/2-1* desiccation experiment would suggest that the ability of the plant to respond to external stimuli is not compromised, however, as the level of response was not quantified it may not be responding in a fully WT-like manner. Alternatively, it is possible that the lack of cuticular ridge effects the ability of the stomata to fully close; again, this could also be investigated using ABA as a trigger for stomatal closure (Schroeder, Kwak and Allen, 2001) and observing the results under the microscope.

To understand the impact of the absent cuticular ledge seen in these mutants, further investigation is required. In the images taken to investigate the lack of cuticular ledge of these mutants it appears that the stomata themselves are larger in size. The TEM and cross section images (Figures 3.15, 3.16, 3.17 and 3.18) indicate that this might be due to the stomata being flatter and less upright causing them to spread out and appear bigger. As RGII dimerisation is known to be important for pore size and rigidity of the cell wall (Fleischer, O'Neill and Ehwald, 1999; Ryden *et al.*, 2003) it may be possible that the lack of RGII dimerisation in these mutants may cause the guard cells to be less able to fully withstand the turgor needed to open the stomatal pore (Hunt *et al.*, 2017). This theory may be supported by results seen in *FOCL1* cell wall mutants thought to be involved in cell wall assembly or maintaining the structure and rigidity of the cell wall. These mutants showed a marked increase in stomatal size when compared to WT and displayed a similar cuticular ledge phenotype to the RGII dimerisation mutants (Hunt *et al.*, 2017). However, the size difference seen between RGII dimerisation

mutants and WT were just initial observations, a full investigation into stomatal size would need to be carried out to assess the effect of RGII on guard cell morphology (Franks and Beerling, 2009). In addition to this, it would also be interesting to investigate the pore size or stomatal aperture of the mutant stomata when fully open using light as a trigger (Shimazaki *et al.*, 2007; Gonzalez-Guzman *et al.*, 2012). Together these experiments could show how the altered stomatal morphology impacts opening and closing of the stomata and in addition would confirm the ability of the stomata to respond to external stimuli. This in turn may be able to explain the desiccation phenotypes observed.

Given the similarities between freezing and drought (Levitt, 1980; Pearce and Fuller, 2001) it is possible that the freezing phenotype may also be explained by the altered stomatal morphology. If the RGII dimerisation mutants are unable to properly close their stomata, it is possible that this could also explain the freezing sensitivity observed in the mutants. Cold tolerant plants normally respond to periods of low temperature by closing their stomata, a response that is not seen in cold sensitive plants (Wilkinson, Clephan and Davies, 2001). The closure of stomata is thought to help maintain water potentials, but it is possibly also a protective measure against freezing as ice may be able to enter leaves through open stomata. Ice could then form a nucleation point within the leaf leading to higher levels of damage and, in extreme cases, death (Pearce, 2001). While it has not been shown that ice enters leaves via stomata more readily in RGII dimerisation mutants, increased pathogen entry via stomata has been seen in *scord6* mutants. As the *MUR1* mutants *sfr8* and *bor1* also display the same absent cuticular ledge it is possible that the same defect that allows pathogens to enter leaves could also allow ice to enter.

4.8.2 Stomatal density in RGII dimerisation mutants

Another possible explanation for the increased desiccation rates in *sfr8* and *bor1-3/2-1* mutants is that they have higher stomatal density. An increased stomatal density could explain the sharp initial water loss seen in the mutant lines (Hepworth *et al.*, 2015; Bertolino, Caine and Gray, 2019; Caine *et al.*, 2019). When investigating density per cm^2 via SEM images it was seen that *sfr8* and WT leaves showed a very similar stomatal density both with and without a period of acclimation (Table 3.1). However, acclimated plants show fewer stomata per unit area. This may be due to the leaf growth that occurred during the 2-week period of acclimation as it has been hypothesised that stomatal size may increase with leaf expansion to account for the reduced number of stomata per unit area (Sack *et al.*, 2003; Carins Murphy, Jordan and Brodribb, 2012). In addition, both *bor2-1* and *bor2-2* showed a very similar density to non-acclimated WT leaves. However, *bor1-3* showed almost twice as many stomata per cm^2 while *bor1-3/2-1* showed approximately three times as many stomata per cm^2 as WT. This

suggests that increased stomatal density could be responsible for the rapid water loss seen in *bor1-3/2-1* mutants, however, the increased water loss seen in *sfr8* cannot be explained by stomatal density. As such, the increased density seen in *bor1-3* and *bor1-3/2-1* plants cannot be explained by the lack of RGII dimerisation in these mutants as *sfr8* shows a WT-like phenotype. This suggests there may be multiple factors influencing *bor1-3/2-1* desiccation, some of which do not involve defective RGII dimerisation.

While stomatal density indicated an increased number of stomata in *bor1-3* and *bor1-3/2-1* mutants, it does not take into account the ratio of epidermal cells to stomata. Considering this ratio by calculating stomatal index can give a far clearer indication of stomatal distribution (Schoch, Zinsou and Sibi, 1980). In this case, alterations to the epidermal cells of the leaf could be seen in *bor1-3* and *bor1-3/2-1* leaves. Cells appeared much smaller and less expanded in the mutant leaves when compared to WT or *sfr8* (Figure 3.19). Unfortunately, stomatal index could not be calculated in this instance due to both the quality of the images taken and time restrains when carrying out experiments. In the future, investigating stomatal index further using confocal imaging of leaf impressions would produce clearer images and be a more effective method to resolve stomatal index (Geisler, Nadeau and Sack, 2000). However, it is believed that stomatal density may change with leaf expansion and that stomatal size can vary with environmental conditions to influence gas exchange (Bertolino, Caine and Gray, 2019).

The small size of the epidermal cells in *bor1-3* and *bor1-3/2-1* leaves would suggest that the lack of *BOR1* is resulting in a cell expansion phenotype in these mutants. This may indicate that boron is required in leaves for epidermal cell expansion as when supplemented with BA the epidermal cells displayed WT-like expansion (not shown). However, these are very provisional observations and further investigation would need to be carried out to quantify the epidermal cell size of the mutant lines with and without BA supplementation. This could be a very interesting line of investigation to follow up in the wake of recent speculation about whether, as previously thought, boron is actually required for plants growth, development and reproduction (Lewis, 2019).

The results from the desiccation and stomatal investigation experiments would suggest that RGII dimerisation plays a role in the desiccation response. The single leaf and whole plant desiccation experiments on *bor1-3/2-1* and *sfr8* (Figure 3.12, 3.13 and 3.14) suggested this however, as discussed, the size variation between *bor1-3/2-1* and WT and *sfr8* presented some challenges. Investigating stomata on a cellular level provided a more direct comparison between *bor1-3/2-1, sfr8* and WT. The lack of cuticular ledge observed in the RGII dimerisation mutants (Figures 3.15. 3.16, and 3.18) suggests that the phenotype is due to a lack of RGII dimerisation. This is supported by the fact that supplementation of *bor1-3/2-1* with BA was

able to partially restore a WT-like phenotype (Figure 3.17). As discussed above there are several experiments that need to be carried out before this stomatal phenotype can be directly linked to altered desiccation. However, the correlation between the missing cuticular ledge and altered desiccation observed in the RGII dimerisation mutants would suggest the two are linked.

4.9 Conclusions

An increase in freezing sensitivity has been seen in *bor*, *sfr8* and *mur1* mutants. These mutants all display defective RGII dimerisation suggesting dimerisation is required for plant freezing tolerance. This is supported by data showing BA supplementation of *bor1-3* and *bor1-3/2-1* restores both RGII dimerisation levels and freezing tolerance. Similarly, fucose supplementation restores RGII dimerisation in *sfr8* mutants and has previously been shown to restore *sfr8* freezing sensitivity (Panter, 2019). All mutant lines investigated indicate that lack of RGII dimerisation impacts innate freezing tolerance rather than the plants ability to acclimate, which suggests *sfr8* may have been incorrectly identified as a cold-acclimation mutant.

It also appears that RGII dimerisation plays a role in stomatal function and development. Desiccation experiments remain slightly unclear due to variation in leaf size and possible boron toxicity. However, results suggest that both *sfr8* and *bor1-3/2-1* have increased rates of desiccation, which is improved with BA supplementation. In addition, thermal imaging experiments on *bor1-3/2-1* mutants suggest that response to external stimuli may not be affected and that instead stomatal morphology, density or ability to close may be affected. When investigated *sfr8, bor1-3* and *bor1-3/2-1* all showed an altered stomatal phenotype displaying a lack of cuticular ledge. As such, this is thought to be linked to the defective RGII dimerisation observed in these mutants and may be responsible for the altered desiccation seen in leaf drying and thermal imaging experiments. Supplementation of *bor1-3/2-1* with BA restored a WT-like phenotype, which could explain the restored desiccation response seen upon supplementation. However, further experiments would be required to fully determine the role of RGII dimerisation in stomatal morphology and behaviour.

In addition, *bor1-3* and *bor1-3/2-1* were seen to have significantly higher stomatal densities than either *sfr8* or WT, which may contribute to the increased desiccation rates observed in these mutants. However, epidermal cell expansion also appeared to be affected in these mutants, suggesting boron may be required for cellular expansion as this was not seen in BA supplemented *bor1-3/2-1*. As this was not seen in *sfr8* mutants RGII dimerisation does not

appear to be involved. However, further investigation would be required to confirm a role for boron in cellular expansion.

These results highlight the importance of the cell wall and, in particular, RGII dimerisation in freezing and drought tolerance. As such, this may be an interesting area for further investigation with regard to development of freezing and drought tolerant crops. In addition, these results indicate the importance of boron as a key nutrient for plant growth and development.

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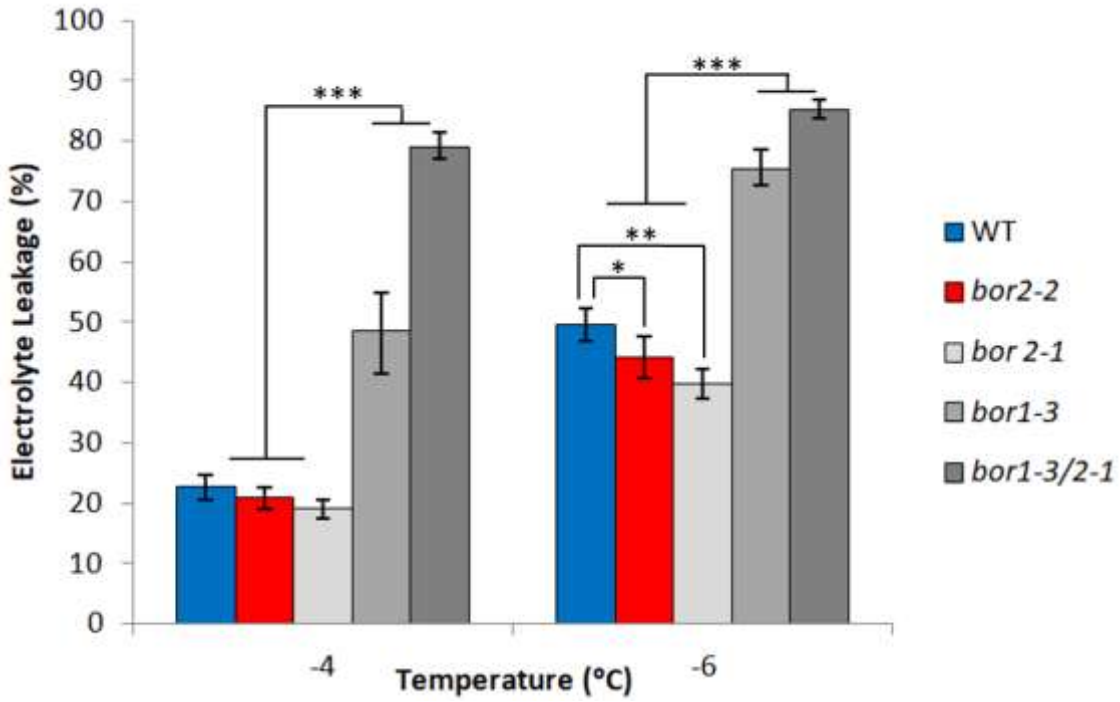
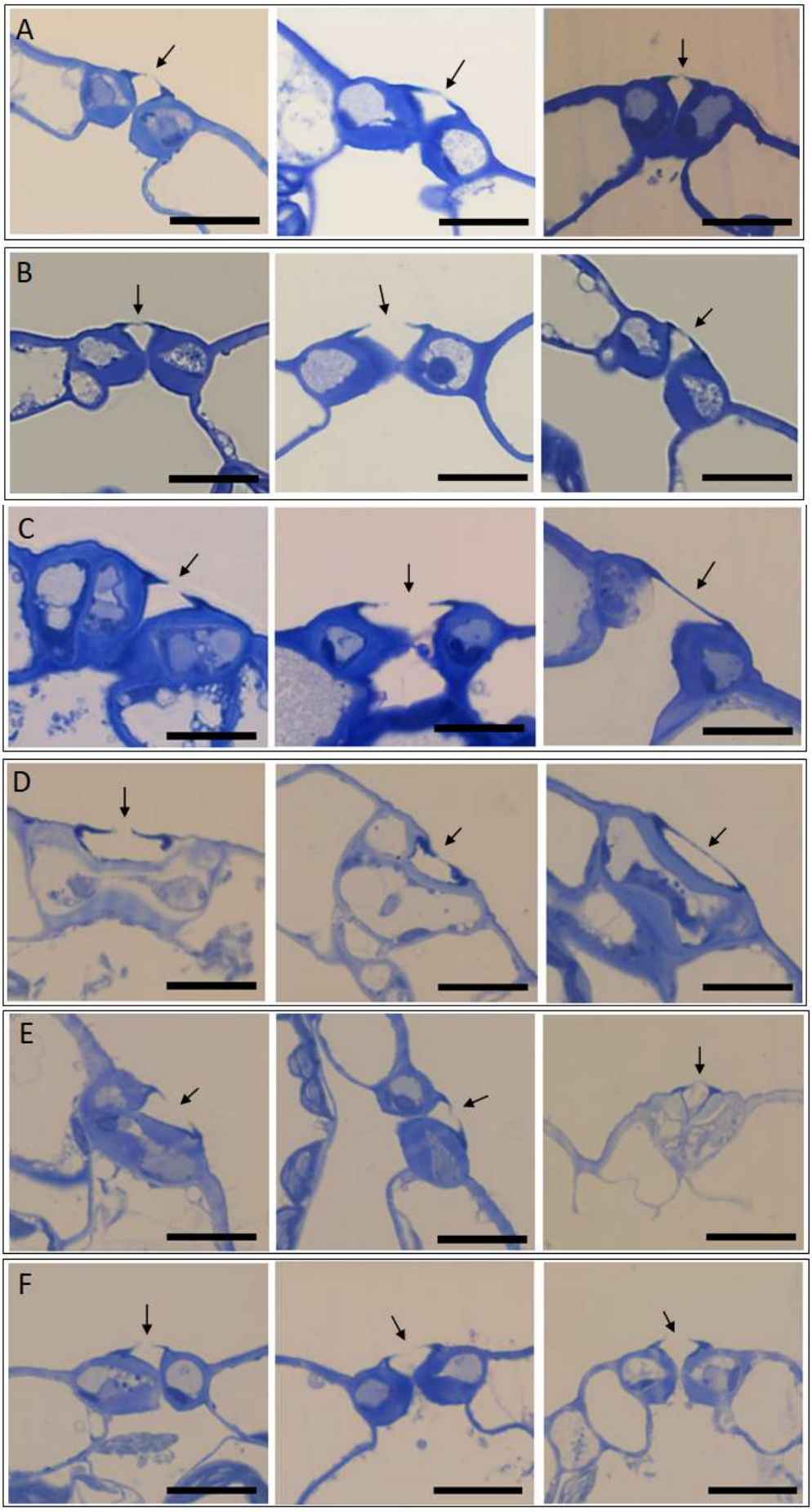
APPENDIX A

Electrolyte leakage levels from whole leaves of WT and *bor* mutant plants including *bor2-2*. Values indicate percentage of electrolytes lost from leaves when exposed to -4°C and -6°C. Results are an average of 2 biological replicate experiments. Six replicate tubes, each containing 3 leaf discs were used for each genotype and temperature per experiment. Results were arcsine transformed and analysed by least-squares means (LSM) comparison at each temperature (one-way ANOVA/LSM ***, *P* <0.001). Error bars represent +/- 1 SE calculated from arcsine-transformed data.



APPENDIX B

Light microscopy cross-section images of stomata from 5-week-old plants. Three images have been shown per genotype to illustrate the variation in morphology for each genotype **A)** WT, **B)** *sfr8*, **C)** *bor1-3/2-1,* **D)** *bor1-3*, **E)** *bor2-1*, **F)** *bor2-2.* Images were taken at 100x magnification a scale bar of 100 µm is shown. Arrows indicate the stomatal pore and the cuticular ledge can be seen on either side.


A
B
C
D
E
F