Expression and functional characterisation of genes putatively involved in freezing tolerance in Arctic species of Vaccinium.

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Expression and functional characterisation of genes putatively involved in freezing tolerance in Arctic species of *Vaccinium*.

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Statement of Authorship

All of the work described in this thesis is my own original research unless otherwise acknowledged in the text or references, and has not been submitted for a degree in this or any other university.

Dedication

My thesis is dedicated to my grandma Mary Ellen Franey who was excited about me starting my masters at Durham University, but never got to see me finish with a PhD.

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Rachael J Oakenfull

Freezing temperatures are responsible for destroying vast areas of crops globally each year and are a major factor in explaining the size and distribution of plants. The ability of plants to survive freezing events, depends on predictable and gradual lowering of temperatures, allowing cold acclimation to occur before freezing temperatures arrive. Climate change is altering maximum and minimum temperatures globally and, making freezing events less predictable. A further understanding of the molecular mechanisms of acquired freezing tolerance would give insights into potential genetic targets for crop improvement. In *Arabidopsis*, CBF/DREB1 transcription factors are currently the most well characterised proteins associated with acquired freezing-tolerance.

Studies were performed on three species of *Vaccinium* collected from the Arctic, *V. myrtillus*, *V. uliginosum* and *V. vitis-idaea*. Genes encoding CBF/DREB1 transcription factors from each of these Arctic species were isolated, sequenced and expressed in *Arabidopsis*. Characterisation studies were performed on transgenic lines generated from each of the three *Vaccinium* species. Induced *COR* gene expression, freezing tolerance and altered phenotype were measured in these lines.

Results showed that *V. myrtillus CBF/DREB1* can induce freezing tolerance in *Arabidopsis.* Western blot and reporter gene assay analysis of transiently-expressed *CBF/DREB1* from *Vaccinium*, highlighted the possibility that the CBF/DREB1 from *V. uliginosum* was less stable than the CBF/DREB1 from the other two species. Site-directed mutagenesis of five regions of interest between the three *Vaccinium* species showed that the substitution of two amino acids improved *COR* gene binding/induction. This substitution could serve as a potential crop improvement site for commercial blueberry crops (*V. corymbosum*).

Other genes associated with the CBF/DREB1 pathway in *Arabidopsis* were cloned and sequenced from the three *Vaccinium* species. Cold-induced expression of these genes was tested and showed similarities in sequence and expression pattern between *V. vitis-idaea* and *Arabidopsis.*

List of Abbreviations

Standard amino acid codes and chemical element signs and abbreviations have been used throughout.

Standard conventions for gene and protein names have been used; genes names are italicised and protein names non-italicised.

Standard unit abbreviations have been used for molarity (µM, mM, M), time (s, min, h), temperature (^{o}C), weight (ng, µg, mg, g) and volume (µl, ml).

Other abbreviations used are as follows:-

ABA - Abscisic acid

ABRE - ABA responsive element

AP2 (transcription factor) - Activation protein 2

AP2/EREBP - Apetala2/ethylene responsive element binding protein

A. tumefaciens - *Agrobacterium tumefaciens*

BASTA - Phosphinothricin (herbicide)

bHLH - Basic helix-loop-helix

CBF - C-repeat binding factor

cDNA - Copy DNA

COR - Cold on responsive

CRT - C-repeat element

DEPC - Diethylpyrocarbonate

DRE - Dehydration responsive element

DREB1 - Dehydration responsive element binding protein 1

EcaICE1 - *Eucalyptus camaldulensis* inducer of CBF expression 1

E. coli - *Escherichia coli*

ERF - Ethylene responsive factor

ESK1 - Eskimo

EST - Expressed sequence tag

FRAP - Fluorescence recovery after photobleaching

GA - Giberellic acid

GFP - Green fluorescent protein

GOLS - Galactinol synthase 3

HOS - High expression of osmotically responsive

HSP - Heat shock protein

ICE - Inducer of CBF expression

KIN - Cold inducible

LB - Luria Bertoni

LTI - Low temperature inducible

mdclbHLH - *Malus domestica* CBF

mRNA - Messenger RNA

MS - Murashige and Skoog

MYB15 - Myelablastosis transcription factor 15

MYB - Myelablastosis element

MYC - Myelocytamatosis element

N. benthamiana - *Nicotiana benthamiana*

P5CS - Δ1-pyrroline-5-carboxylate synthetase

OSICE - *Oryza sativa* inducer of CBF expression

PCR - Polymerase chain reaction

qPCR - Quantitative PCR

R2R3 - Repeat 2 repeat 3

RACE PCR - Rapid amplification of cDNA ends PCR

RNAi - RNA interference

RuBisCO - Ribulose-1,5-bisphosphate carboxylase/oxygenase

SA - Salicylic acid

SDS - Sodium dodecyl sulphate

SIZ1 - Small ubiquitin-like modifier E3 ligase

SLU - Sveriges Lantbruksuniversitet - Swedish University of Agricultural Sciences

TaICE - *Triticum aestivum* inducer of CBF expression

TE buffer - Tris EDTA buffer

TIFF - Tagged image file format

UPSC - Umeå Plant Sciences Center

V. myrtillus - *Vaccinium myrtillus*

V. uliginosum - *Vaccinium uliginosum*

V. vitis-idaea - *Vaccinium vitis-idaea*

wt - Wild type

Chapter 1 Introduction

1.1 Climate change

Climate change is defined as a significant change in the climate lasting for an extended period of time, therefore including any major changes in temperature, wind patterns or precipitation that occur over several decades or longer (www.epa.gov/climatechange).

Climate change is affecting food supplies as such change directly impacts crop production. The knock on effects of which influence markets and food prices and therefore the food supply chain (Gregory et al., 2005). Food prices are already increasing globally each year and will continue to increase due to factors such as oil prices and the use of agricultural land for biofuel production. This will eventually make food unaffordable for many countries (Chand, 2008). The world food programme estimates that by 2050 an extra 20% of the population will go hungry due to climate change (www.wfp.org).

1.2 How is climate change affecting plants

Climate change is altering the maximum and minimum temperatures across many areas of the globe, meaning that crop growth in some areas is becoming increasingly difficult and large areas of crops are lost to unfavourable weather conditions every year. Examples of crops being lost to freezing temperatures over a number of years include Israel losing citrus crops (www.jta.org), Mexico losing 90% of its maize crop, which in turn caused food prices to triple (www.martellcropprojections.com, www.earthobservatory.nasa.gov), and Zimbabwe (a major exporter of flowers) losing large amounts of flower and vegetable crops (www.thezimbabwean.com). More recently, in 2011 India suffered its coldest winter in 30 years, destroying huge areas of crops (www.ntd.tv) and in 2012 North America lost large areas of citrus crops (www.westernfarmpress.com). In 2013 blueberries, blackberries and vegetables were damaged in California during the long cold spell endured over an unusually harsh winter that year (www.sanluisobispo.com).

1.3 Importance of understanding freezing tolerance

Many important staple food crops, such as wheat, barley, rice and oilseed rape, can be damaged by freezing temperatures (Chinnusamy et al., 2007). As climate change progresses, it is thought that some areas currently used for crop cultivation will become unusable for the crops currently grown there. For example, areas currently used for citrus growth may in the future have to be used for crops that are more chilling/frost tolerant and new growing areas found (Parmesan & Yohe, 2003). However, substituting the type of crop

grown in each area may not always be possible and some areas may become totally unusable due to the more extreme growth and survival conditions faced (Thomas et al., 2004, Thomashow, 1999). Using crops such as winter wheat (*Triticum aestivum* cultivar Horatio) is a step towards solving part of this problem as the seed can be sown in autumn and the crop grow/survive over winter and then harvested the following summer (Brulebabel & Fowler, 1988). Although this could be a potential answer to reducing wheat loss in areas that experience a spring frost, the growth time needed is *ca.*12 months, therefore requiring a year of farming and maintenance to obtain the same amount of grain as produced from spring wheat over a six month period (www.ukagriculture.com).

It is possible to improve food crops by genetic modification. This has been demonstrated through a number of projects, such as the production of 'golden rice' using the addition of *psy* genes from wheat to enhance the nutritional content of *Oryza sativa* (rice) crops (Paine et al., 2005). Another example is increasing the level of antioxidants produced in *Lycopersicon esculentum* (tomato) crops to improve their health benefit. This was achieved by expressing transcription factors from *Antirrhinum majus* (snapdragon) in *L. esculentum* causing the accumulation of anthocyanins (Butelli et al., 2008). Crop plants have also been manipulated for pest resistance and improved shelf-life.

Genetic modification has been used successfully to increase freezing tolerance in *Solanum tuberosum* cv. Umatilla (potato) crops by expressing *CBF/DREB1* (See section 1.8.5) from *Arabidopsis*. The ability to cold acclimate was improved, thereby increasing the freezing tolerance of the potato crop by 2^{o}C, taking the freezing tolerance down to -5^{o}C. As the *CBF/DREB* genes were induced by a stress-specific promoter, there were no detrimental effects on growth and tuber production under normal conditions (Pino et al., 2007).

A better understanding of freezing tolerance in plants could stem from identification of new genes that are involved in freezing tolerance, or versions of *CBF/DREB1* that could potentially induce improved freezing tolerance. This would allow further crop improvement to produce more freezing-tolerant crops, and therefore reduce the number of crops lost globally each year to frost (Sanghera et al., 2011).

1.4 Freezing damage and acclimation- how do plants survive cold?

Low temperature tolerance is a major factor in determining the geographical distribution of crops (Knight & Knight, 2012). Plants can be split into three types: chilling sensitive; chilling tolerant; and freezing tolerant. Chilling sensitive plants are not capable of cold acclimating (Hannah et al., 2005), and are therefore damaged by temperatures below +10^{o}C, (Levitt J., 1980). Chilling sensitive crops include: rice; soybean; maize; cotton; and tomato, the latter

is damaged at temperatures of +12^{o}C (Vega-Garcia et al., 2010). Chilling tolerant plants can withstand low positive temperatures but are damaged by freezing temperatures (Chinnusamy et al., 2007). Freezing-tolerant plants can survive temperatures as low as -40^{o}C (Thomashow, 1998) by cold acclimating to avoid freezing-induced damage (Thomashow, 1999, Xin & Browse, 2000). Freezing-tolerant crops include *Secale cereal* (winter rye) (Antikainen & Griffith, 1997), *Triticum aestivum. Horatio* (winter wheat) (Brulebabel & Fowler, 1988) and *Solanum commersonii* (wild potato) which can survive temperatures of -11^{o}C (Chen & Li, 1980). In extreme cases, boreal trees such as *Larix* spp. (larch) and *Populus* spp. (poplar) can survive freezing below -40^{o}C and can survive freezing in liquid nitrogen (Sakai & Larcher, 1987).

The two main ways in which freezing can damage plants are through cellular dehydration or membrane damage; both occuring due to the formation of ice crystals (Thomashow, 1999). As ice has a more negative water potential than water, ice crystals in the intercellular compartments grow by drawing water out of the cytoplasm and into the intercellular space, causing increasing damage as the ice crystals grow, as well as causing cytoplasmic dehydration of the cell, potentially leading to cell death (Lissarre et al., 2010, Pearce, 2001). When thawing occurs, the water then moves back into the cytoplasm, this may cause the cell to burst ("freeze-induced lysis") if the previous severe dehydration caused the protoplast to shrink, therefore meaning that the cell can no longer hold the same volume of water as before exposure to freezing temperatures (Uemura & Steponkus, 1989). Membrane structures are also damaged by freeze-induced dehydration; this results in a loss of compartmentation, allowing leakage of electrolytes and compatible solutes even before thawing occurs (Pearce, 2001). Freezing can cause protein denaturation (Guy et al., 1998), and has even been reported to cause tree trunks to split by increasing tension in the wood (Pearce, 2001).

Certain plants avoid freezing damage by employing a process of supercooling, whereby water is moved out of the cells, thus increasing the concentration of compatible solutes within the cells and therefore lowering the freezing temperature of the cytoplasm. This also allows water to freeze outside the cells, thus causing less damage. This mechanism, however, cannot be used for prolonged periods as it leads to dehydration which would eventually reach the same level as that induced by ice crystal formation (Pearce, 2001). Other plants, such as poplar, have highly viscous cellular contents, that may transition into a glass vitrification phase to prevent freezing, (Hirsh, 1986). By forming intracellular glasses using compatible solutes, these plants can prevent intracellular ice formation, and

therefore water loss and dehydration, allowing them to become freezing tolerant to around -20^{o}C (Hirsh, 1986). It has also been found that some chilling-sensitive plants can down regulate photosystem two of photosynthesis in response to cold and use some of the energy from light as heat to prevent freezing (Fryer et al., 1998). The cooler temperatures limit the activity of photosynthetic electron sinks causing a reduction in the amount of energy transferred to the light harvesting complex. This then allows extra energy to be used to produce heat preventing further chilling of leaves (Allen & Ort, 2001).

The most commonly studied method plants employ to avoid freezing damage is through cold acclimation. Cold acclimation is the process through which plants acquire (seasonally) increased freezing tolerance; it is often triggered by a combination of shorter photoperiods and a period of low positive temperatures of around +5^{o}C to as low as 0^{o}C (Thomashow, 1999). Depending upon the species, the period required for acclimation can be as short as three days or, for trees for example, as long as a few weeks. However, the full process of cold acclimation is still not fully understood, due to the large number of different mechanisms involved. From the time when the cooler temperatures are first sensed, a long chain of events needs to occur to prepare the plant for freezing, starting with changes in transcription and gene expression (Chinnusamy et al, 2010, Gilmour & Thomashow, 1991, Hannah et al., 2005, Weiser, 1970). In *Arabidopsis* a number of genes involved with protein biosynthesis, lipid metabolism, chloroplast function, free radical detoxification and carbohydrate metabolism were found to be induced in response to cold (Provart et al., 2003). During acclimation growth stops and genes for the production of photosynthetic energy are down regulated (Fowler & Thomashow, 2002); changes are also needed in metabolome, proteome and the composition and structure of membranes and cell walls (Guy et al., 2008). The most studied group of genes that is up-regulated during cold acclimation encodes pathways involved in preventing desiccation and in the accumulation of compatible solutes (Knight & Knight, 2012). When compared to those of freezing sensitive plants, leaves of freezing-tolerant plants were found to contain a number of compatible solutes which potentially protected membranes against inactivation during freezing (Rudolph & Crowe, 1985). Cryoprotectants such as sucrose, proline, serine, succinate (Volger & Heber, 1975), trehalose, fructans and polyols contribute to freezing tolerance in plants (Smirnoff, 1998). Accumulation of these compatible solutes is an important part of acquiring freezing tolerance (Kaplan et al., 2004); by lowering the water potential inside the cytosol they can prevent water loss to ice in the intercellular space, also preventing further ice crystal formation. Cryoprotectants also protect thylakoid

membranes from damage by reducing their permeability to solutes and allowing increased expansion during thawing, thus providing increased resistance against freeze induced osmotic stress (Hincha et al., 1990). The accumulation of soluble sugars as part of cold acclimation in *Arabidopsis* was shown by Strand et al., (1997).

Another problem caused by freezing stress results from low temperatures causing the membranes within plant cells to become rigid (Alonso et al., 1997). During cold acclimation, genes encoding fatty acid desaturases are activated; these desaturases add double bonds to fatty acyl chains of membrane lipids (Murata & Los, 1997) or phospholipids (Anchordoguy et al., 1987) increasing membrane fluidity once again (Murata & Wada, 1995, Nishida & Murata, 1996). This change however, limits the normal physiological functions of the plant cells (Raison & Orr, 1986). Membranes are further stabilised by the production of dehydrins and compatible solutes through either direct interaction with the surface of the membrane or interaction with the surrounding water (Close, 1996, Strauss & Hauser, 1986).

Examples of these processes have been found in a number of different species. Comparisons between the soluble protein content of freezing-tolerant and freezing-sensitive varieties of wheat showed a correlation between the accumulation of soluble proteins and increased freezing tolerance (Perras & Sarhan, 1989). Increased protein synthesis was also seen in *Brassica napus* (rapeseed) in response to low temperatures (Mezabasso et al., 1986). Further, a period of cold acclimation increased the freezing tolerance of *Secale cereale* (rye) protoplasts from -5^{o}C to -25^{o}C as a result of a change in the phospholipid composition of the plasma membrane (Uemura & Steponkus, 1989). The ability to acclimate can make the difference between severe damage and survival for plants. Acclimation comes at a great energetic cost to the plant, however, therefore only occurring when essential to survival (Dobrota, 2007). The process of acclimation takes much longer than that of deacclimation (Arora et al., 1980). As temperatures become more variable due to climate change, (Bokhorst et al., 2011) the ability not to de-acclimate during winter warming, or to re-acclimate quickly, will become essential for survival (Kalberer et al., 2006).

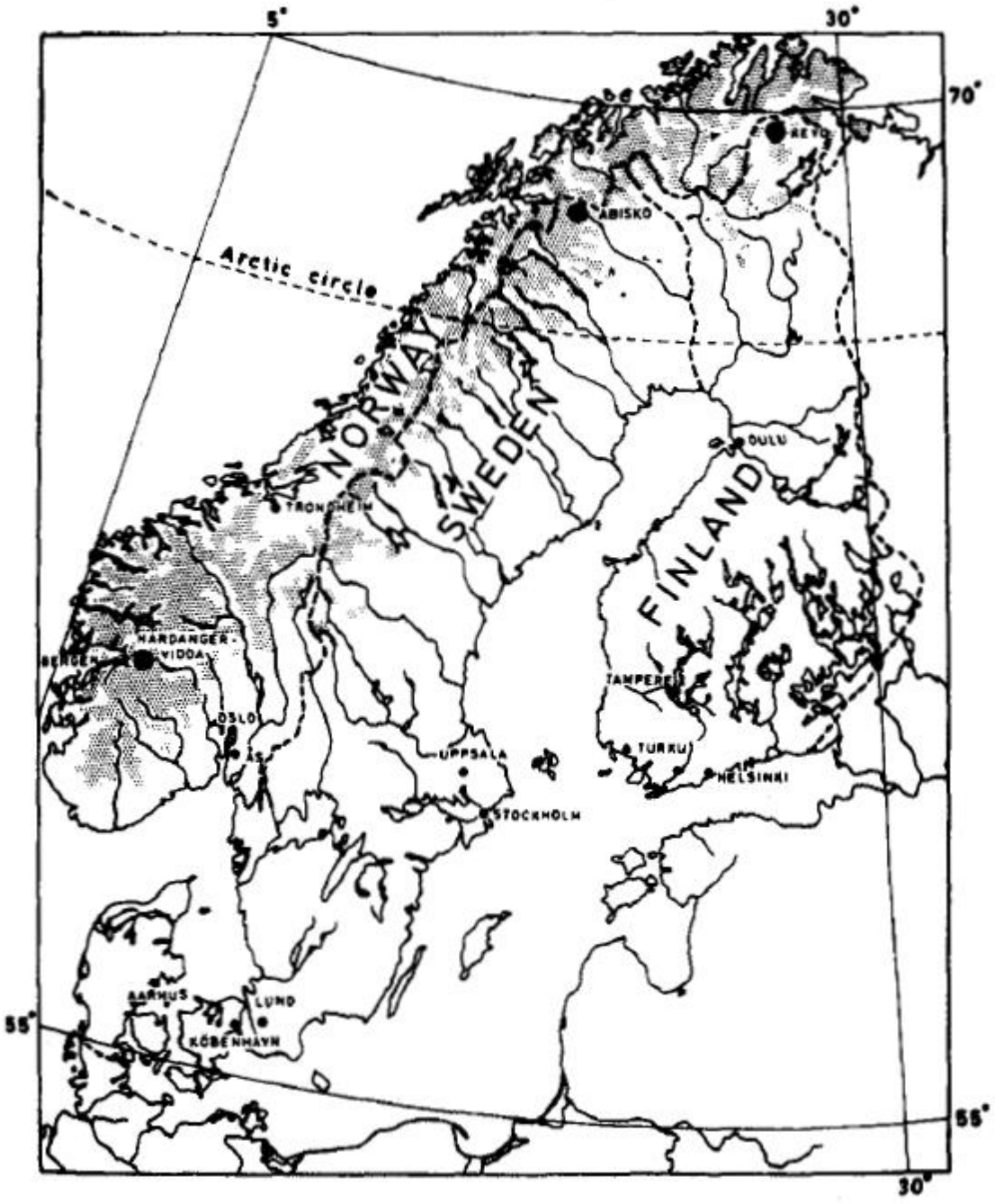

Figure 1.1: A map of the Fennoscandian countries showing the location of Abisko in far northern Sweden. From (Sonesson & Callaghan, 1991)

1.5 Tundra in the sub-Arctic

Half of the carbon dioxide emitted from burning fossil fuels accumulates in the atmosphere; the other half is stored in the oceans and in terrestrial carbon sinks (Bousquet et al., 2000). The sub-Arctic provides a significant carbon sink for carbon dioxide from the atmosphere (Goodale et al., 2002, Turunen et al, 2002). Sub-Arctic regions also play an important role in surface albedo feedback, incoming solar radiation being reflected back into the atmosphere, therefore maintaining cooler temperatures, due to the reflective properties of snow cover (Winton, 2006).

Abisko is situated in the north of Sweden (Figure 1.1, 68°21'N,18°49'E), *ca.* 200 km north of the Arctic Circle (www.polar.se) next to lake Torneträsk (Figure 1.2). Abisko Scientific Research Station is run by the Swedish Polar Research Secretariat and has recorded and monitored climate, physical and biotic conditions in Abisko and the surrounding areas for over 100 years (Jonasson et al., 2012). As much of the area surrounding Abisko is owned by the Sámi this adds another level of information available about the area in the form of Traditional Ecological Knowledge (TEK) observed by them over many years. This includes factors such as plant species growth and availability due to snow depth, and ice cover, especially as it affects Reindeer herding (Riseth et al., 2011).

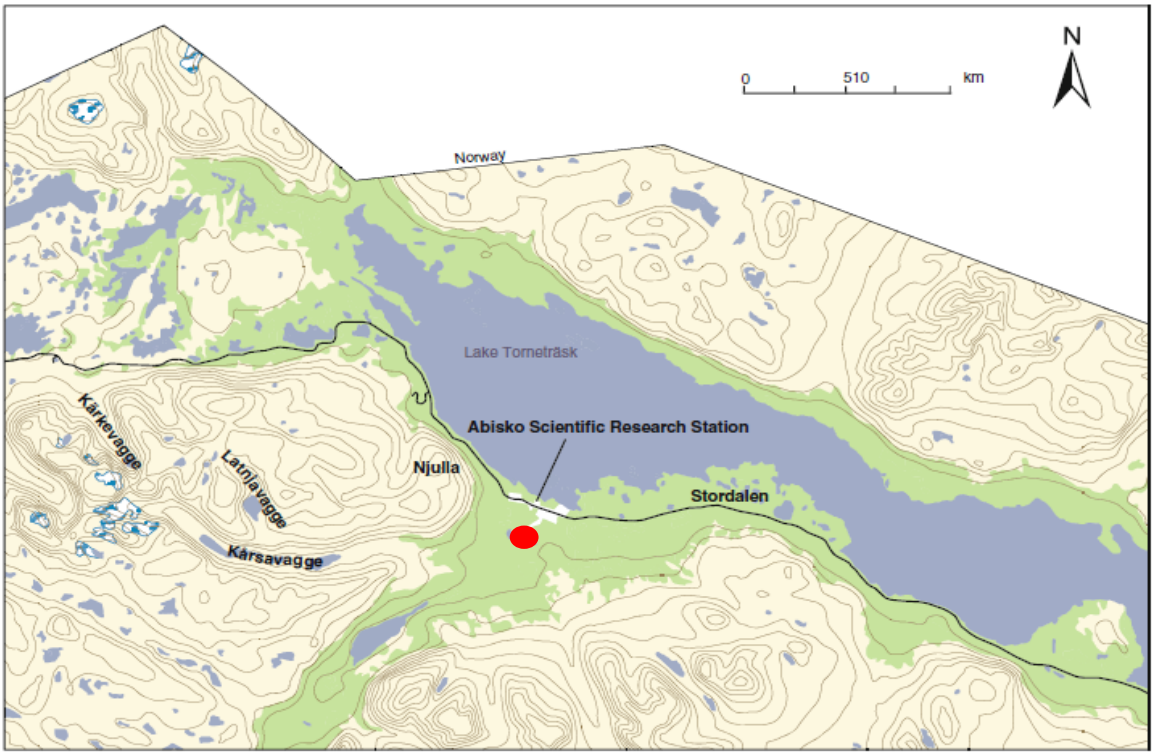

Figure 1.2: Map of Abisko and the surrounding areas showing the location of Abisko Scientific Research Station (ANS). From (Jonasson *et al.*, 2012). Blue shading represents major freshwater bodies; green shading represents forest; black line represents the road E10; grey lines represent elevation contours; red circle indicates the area from which plant samples were taken.

Abisko has an Arctic photoperiod (short daylight hours in winter and no daylight for several weeks midwinter then 24h daylight in mid-summer); the annual mean temperature is -1^{o}C (Sonesson & Lundberg, 1974), with seasonal temperatures fluctuating between -35^{o}C and +20^{o}C (observed). Abisko, despite being in the sub-Arctic, has a climate 1^{o}C warmer than temperatures usually associated with sub-Arctic climates due the relative proximity of the ocean and associated air masses (Andersson et al., 1996). Beside lake Torneträsk there are birch forests and dwarf shrub tundra heaths. As elevation increases the trees become sparser until the ecotonal transitions to tundra heath is reached, comprised principally of dwarf shrub communities and isolated patches of birch trees. Ascending further across this ecotone, into the Alpine zone, the vegetation is dominated by lichens and mosses, with low-growing Arctic Alpine species (Löve, 1970). Arctic summers are short; many plants in the sub-Arctic therefore have a short growth season. Because of this, most of the plants found in the sub-Arctic are perennials, as plant developmental processes are generally extended beyond one growth season. For 8 to 9 months of the year there is snow cover and areas of the ground are frozen until spring (Akerman & Johansson, 2008, Bosio et al., 2012). Tundra soils are disturbed by frequent freeze thaw cycles meaning they are often low in nutrients (Sonesson & Callaghan, 1991). Due to the harsh growing conditions faced by plants in the Arctic, many plants survive by growing in areas such as birch forests and hollows where they will become covered by accumulated snow in the winter months. The snow insulates the plants against the harsh temperatures, as well as protecting them from the winds and early spring desiccation experienced in more exposed areas (Sonesson & Callaghan, 1991, Sonesson & Lundberg, 1974). Plants growing in exposed areas are more commonly evergreen dwarf shrubs as these are better able to survive water loss in late winter when shoots are exposed above the snow pack and are therefore in direct sunlight. This heats the leaves causing them to lose water which cannot be replaced because the ground, and therefore roots and surrounding soil water, are still frozen (Sonesson & Callaghan, 1991). Such plants also have to survive grazing damage to exposed leaves and stems caused by species such as *Rangifer tarandus* (reindeer); *Lemus lemmus* (lemming); *Lepus timidus* (mountain hare) and *Lagopus lagopus* (ptarmigan).

1.6 Climate change and Arctic plants

The Arctic is an important indicator of climate change; factors such as prolonged changes in snow depth and sea ice extent are clear, quickly identifiable, symptoms of change (Weller, 2001). Climate change is one of the most researched topics in Abisko with many groups researching how climate change will affect the sub-Arctic environment. Researchers have

examined, *inter alia*, changes in the fluxes of carbon dioxide and methane, changes occurring in former areas of permafrost, and changes in vegetation and plant growth associated with altered snow depth and changing temperatures (Jonasson *et al.*, 2012).

Temperature has a major effect upon the distribution of Arctic plant species (Phoenix & Lee, 2004). As climate change progresses it is predicted that Arctic temperatures will increase by between 4 and 7^{o}C by 2100, with the greatest warming occurring in winter; precipitation will also increase (ACIA, 2005). The increase in temperature will potentially cause northward shifts in the treeline and in land suitable for agriculture. However this same warming may also allow species currently not found in the sub-Arctic to become invasive in more northern regions. An increase in temperature may also potentially cause other problems, with more frequent insect outbreaks such as those of *Epirrita autumnata* (the autumnal moth). The warmer temperatures over winter may cause a major change in snow and ice conditions over the long Arctic winter, which may have the most significant effect upon plant species (ACIA, 2005). Arctic plants have adapted to function at low temperatures and can therefore respond rapidly to spring temperatures and snowmelt (Bilbrough et al., 2000). This rapid response to warmer temperatures makes Arctic plants potentially more vulnerable to the effects of winter thawing events, when periods of warmer temperatures during winter cause snow melt, subsequently leaving plants exposed to freezing temperatures when the temperatures drop again. Partial snow and ice melt can also cause problems if it exposes parts of plants but the soil and roots remain frozen, leaving the plant at risk of desiccation (Phoenix & Lee, 2004).

Certain Arctic plants are able to respire at temperatures as low as 0^{o}C (Korner, 1999). Winter warming will increase the temperature enough to melt snow and induce respiration. This respiration uses energy, potentially severely depleting carbon stores within the plant for the following growth period, especially because the Arctic photoperiods mean that there will generally not be enough sunlight for photosynthesis to occur during such winter thaw periods. This may limit the chances of survival of some species over the next growth period (Phoenix & Lee, 2004).

1.7 The study species *Vaccinium*

Vaccinium species belong to the Ericaceae plant family (www.ars-grin.gov) and are found across North America, Northern Europe and Northern Asia.

In Scandinavia, berry production is important both economically and environmentally, as *V. myrtillus* and *V. vitis-idaea* constitute large proportions of forest floor vegetation along with *Rubus idaeus* (raspberry), in more southern parts of Scandinavia. Around 500 million Kilograms of berries are harvested annually in Sweden, of which the berries from *V. myrtillus* and *V. vitis-idaea* constitute a large proportion (Kardell, 1980). These co-existing *Vaccinium* species play an important role in the organisation of heath communities in extreme habitats such as sub-alpine heathland (Gerdol et al., 2000).

Three species of *Vaccinium* that occur in the Arctic were used for this project: *V. myrtillus*, *V. uliginosum;* and *V. vitis-idaea.* They can all be found growing in similar areas of the sub-Arctic (Abisko) but are assumed to have different levels of freezing tolerance because they occupy different environments within the landscape and exhibit different strategies for survival (*personal observation by the author*).

1.7.1 *Vaccinium myrtillus*

Vaccinium myrtillus (Figure 1.3) is commonly known as bilberry, whortleberry or European blueberry (www.ars-grin.gov) and is a deciduous rhizomatous shrub (Ritchie, 1956) with green photosynthetic younger stems, woody older stems and small pink bell shaped flowers (*personal observation by the author*), that are followed by purple/black edible berries (nccam.nih.gov). *V. myrtillus* is native to northern areas of Europe, Asia and North America and is found on bog land and heaths (Ritchie, 1956).

Figure 1.3: *Vaccinium myrtillus*

V. myrtillus is chamaephytic in growth habit (a woody plant whose resting buds are on or near the ground). Since chamaephytic plants overwinter under snow to protect them from harsher environments, the distribution of *V. myrtillus* is determined by the availability of snow cover to shelter it from more extreme conditions. In the sub-Arctic *V. myrtillus* is most frequently found in birch forests; because of its need for shelter *V. myrtillus* is also thought to be tolerant of low light levels (Ritchie, 1956).

V. myrtillus is used as a food in many countries and is also a food source for a number of animals (Fernández-Calvo & Obeso, 2004). Berries from *V. myrtillus* have been shown to have many health benefits due to the high levels of anthocyanins present, these acting as antioxidants (Faria et al., 2005). Studies have shown that antioxidants from *V. myrtillus* may inhibit cancer cell growth (Katsube et al., 2003), as well as playing a role in vasoprotection and as an antioedema treatment (Lietti et al., 1976). Leaves from *V. myrtillus* have been used for a number of years as a traditional treatment for diabetes, due to the compound myrtillin (Allen, 1927) which is one of the many anthocyanins found in *V. myrtillus*.

1.7.2 *Vaccinium uliginosum*

Vaccinium uliginosum (Figure 1.4) is also known as bog whortleberry and bog blueberry (www.luontoportti.com). *V. uliginosum* is a deciduous rhizomatous shrub (Jacquemart, 1996) with woody stems and dark pink bell shaped flowers that are followed by dark blue/ black berries (*personal observation by the author*). *V. uliginosum* is found growing on acidic heaths and bogs in Northern areas of Europe, Asia, and North America. The limiting factor for distribution of *V. uliginosum* across more southern locations is the maximum summer temperature (Conolly, 1970). *V. uliginosum* is thought to be temperature limited to more northern growth areas than *V. myrtillus* and *V. vitis-idaea*. *V. uliginosum* is less studied than the other two *Vaccinium* species, possibly because, although edible, the berries from *V. uliginosum* are not commonly used as a food source, therefore making *V. uliginosum* not as economically important as *V. myrtillus* or *V. vitis-idaea*, and thus not as well characterised.

Figure 1.4: *Vaccinium uliginosum*

1.7.3 *Vaccinium vitis-idaea*

Vaccinium vitis-idaea (Figure 1.5) is also known as mountain cranberry, cowberry and lingonberry (www.luontoportti.com). It is an evergreen shrub whose leaves have a thick glossy upper surface and a pale spotted lower surface (Ritchie, 1955). *V. vitis-idaea* has pale pink/white bell shaped flowers that are followed by edible red berries (*personal observation by the author*). *V. vitis-idaea* is found in mountain and upland heaths, coniferous forests and bogs. Maximum summer temperature is thought to be the limiting factor in the distribution of this species, meaning that *V. vitis-idaea* is found in Northern areas of Europe, Asia, and North America. *V. vitis-idaea* is mostly chamaephytic in growth habit and so snow plays an important role in its survival (Ritchie, 1955). However, it is thought that buds can survive minimum temperatures of between -25^{o}C and -32^{o}C; *V. vitis-idaea* thus could potentially survive without snow cover in some areas (Raatikainen, 1988). Although an evergreen shrub, leaves of *V. vitis-idaea* are thought to lose up to two thirds of their original photosynthetic capability during the second growth season, losing up to a further 10% in each following growth season (Karlsson, 1992).

Figure 1.5: *Vaccinium vitis-idaea*

The fruit of *V. vitis-idaea* is an economically important crop in Sweden and Finland and has been proven to have health benefits. The berries have been shown to aid the prevention of obesity related disorders caused by high fat diets, (Heyman et al., 2014). Lingonberries have high levels of vitamin E, C and manganese, as well as high levels of anthocyanins that are antioxidants with potential medical uses. They are also used as natural preservatives due to the high levels of benzoic acid present (www.arktisetaromit.fi).

1.7.4 *Vaccinium* survival strategies

In some tundra and forest areas of the sub-Arctic all three *Vaccinium* species co-exist; for two species of *Vaccinium,* co-existence with the other species aids their survival. For both *V. uliginosum* and *V. myrtillus* the cover of the other species has a positive effect on growth; both species had reduced growth following the removal of neighbouring different *Vaccinium* species (Shevtsova et al., 1995). However, *V. vitis-idaea* has limited growth in areas where it is found in close proximity to other *Vaccinium* species. This may be because of the slightly different ways the three species grow; growth in all three species varies each year and there is a difference in yearly growth between deciduous and evergreen species (Gerdol *et al.*, 2000).

There are a number of ways that Arctic plants are adapted to survival in low temperatures. All three *Vaccinium* species are perennial and rhizomatous allowing them to store the majority of their biomass, including carbohydrates and lipids, below ground where the temperature is not as low over winter. This also functions during vegetative reproduction in poor growth seasons (Billings & Mooney, 1968, Jacquemart, 1996, Ritchie, 1955, Ritchie, 1956). *V. myrtillus* and *V. uliginosum* invest a higher proportion of biomass in their stems each growing season than *V. vitis-idaea* (Parsons et al., 1994). *V. myrtillus* is capable of photosynthesising at low light intensities, an adaptation commonly found in Arctic plants due to high chlorophyll content in leaves (Billings & Mooney, 1968, Ritchie, 1956). This is something which also assists the rapid increase in photosynthesis in Arctic plants in response to favourable conditions of light and temperature (Billings & Mooney, 1968). This allows the plants to maximise their efficiency during short optimal growth seasons to allow energy storage to begin (Johansson, 1975), which is important in deciduous plants such as *V. myrtillus* and *V. uliginosum*. Certain Arctic species are able to become dormant over winter, triggered by changes in photoperiod and temperature. This dormancy is controlled by temperatures below 0^{o}C (Billings & Mooney, 1968). It is possible that this is linked to the fact that some Arctic plants have stomatal conductance that is controlled by root temperature. If the roots become frozen by temperatures dropping below 0^{o}C, photosynthesis and respiration will not be able to occur as the stomata will be closed (Starr et al., 2000). Further Arctic species are able to initiate rapid growth even at low temperatures; although this may be of importance to all three *Vaccinium* species, it is potentially more important for *V. vitis-idaea* as evergreen shrubs start growth later in the growing season and can continue growing until late summer, since they can recycle photosynthetic components from old leaves, whereas deciduous plants are unable to do this (Karlsson, 1985). *V. vitis-idaea* can compensate for winter energy usage by beginning

photosynthesis in early spring when partial snow melt provides enough water and allows light to pass through the remaining snow. Under snow, *V. vitis-idaea* maintains 25% of its maximal photosynthetic capability without altering its soluble sugar concentration over winter (Lundell et al., 2008). This can only occur, however, if the snow depth permits enough light to pass through. This allows *V. vitis-idaea* rapidly to reach maximum photosynthetic capacity shortly after complete snow melt (Kudo, 1991). It is thought that woody plants can acclimate using a decrease in temperature as a signal regardless of the photoperiod (Weiser, 1970). The ability to cold acclimate efficiently to withstand the low winter temperatures is the most essential adaptation for survival in Arctic conditions (Billings & Mooney, 1968).

1.7.5 Effects of climate change and *Vaccinium*

Studies on the effects of climate change on snow melt timing and soil thaw have shown that the two factors are not always related (depending on the length of the period of warming), thereby making predictions about exact conditions induced by climate change in the Arctic difficult (Van Wijk et al., 2003). Climate change will cause warmer winter temperatures and increased precipitation, therefore altering the snow and ice conditions (ACIA, 2005). The change in ice and snow coverage areas also means that temperatures of some areas will become far more variable (Callaghan et al., 2011, Rinke & Dethloff, 2008). Although it is unknown whether the increased precipitation will be as rain or snow, heavy winter rain or snowmelt may result in plants becoming encased in ice (Hansen et al., 2013, Phoenix & Lee, 2004). However, both *V. myrtillus* and *V. vitis-idaea* showed no significant damage in their growth season following ice encasement (Preece & Phoenix, 2013). The increase in temperature could also lead to more rain followed by periods of snow, as opposed to just snow alone in winter. This can lead to plants underneath snow packs being damaged by snow mould (Bokhorst et al., 2012a). Increased rain may result in shallower snow depths and therefore an earlier snowmelt. Early melt times favour tall, slow-growing plants. High temperatures after snowmelt have been shown to shorten the following growth period of a plant (Jonas et al., 2008), as warmer temperatures have been shown to cause an earlier peak in growth rather than an increase in the overall biomass gain of a plant (Rumpf et al., 2014). Snow depth and coverage is one of the most important factors in determining growth season length, and therefore survival, of Arctic plants (Rumpf *et al.*, 2014, Wipf & Rixen, 2010). Increased snow depth will increase the winter temperatures for plants underneath, potentially causing increased winter respiration and therefore a loss in carbon stores due to the inability to photosynthesise under snow (Morgner et al., 2010,

Walker et al., 1999). Deeper snow will take longer to melt in the following spring and in areas such as sub-Arctic tundra, where the growth season is already short, delays in snowmelt will have significant effects upon plant growth and development (Cooper et al., 2011). Species that have shown the greatest change in growth and development in response to changes in snowmelt are dwarf shrubs such as *Vaccinium* spp. (Wipf & Rixen, 2010). Changes in snow depth will not only change the temperatures under the snow but also the amount of water available. Deeper snow has been shown rapidly to change the species composition in these area because of the warmer winter conditions, cooler summers and an excess of water, which has been shown to favour species such as *V. uliginosum* that will not be negatively affected by the very wet conditions caused *(Scott & Rouse, 1995)*.

Although winter warming events have been recorded every 7 to 10 years in the Abisko region (Bokhorst et al., 2008, Bokhorst et al., 2009), the frequency of warm air masses over regions of the Arctic in winter has increased (Visbeck et al., 2001) and short winter warming events are predicted to become more prevalent as climate change progresses (ACIA, 2005). Short winter warming events are generally considered to be a period of less than 10 days when temperatures rise rapidly to positive temperatures around +5^{o}C causing snow to melt (Callaghan et al., 2004, Phoenix & Lee, 2004). The warmer winter temperatures may induce effects such as bud burst in some species which will then be badly damaged when freezing temperatures return, thus affecting vegetative growth from these buds in the next growing season (Bokhorst *et al.*, 2008, Bokhorst *et al.*, 2009). This is due to the snow melt exposing plants to much colder freezing temperatures than faced under the snow, as well as winter desiccation, wind abrasion and repeated freeze-thaw cycles which would not usually occur under the protective snow cover (Sonesson & Callaghan, 1991).

It is thought that deciduous dwarf shrubs will be more susceptible to winter warming events than dwarf evergreen shrubs. Warmer temperatures will cause bud burst in deciduous species to produce new leaves; the following freezing temperatures will damage the buds/ new leaves, causing a delay in new leaf production in spring or a reduction in the leaves produced in the next growth season, and therefore in the amount of energy produced (Bokhorst et al., 2012b, Van Wijk et al., 2004). Of the three species of *Vaccinium*, *V. myrtillus* is thought to be the species that will be most affected by climate change (Bokhorst et al., 2010), whereas *V. uliginosum* so far has shown little or no response to winter warming events (Bokhorst *et al.*, 2012a). *V. myrtillus* under normal conditions has an

early bud burst to achieve flowering as rapidly as possible in spring (Kudo & Suzuki, 2002). It is possible that short warm spells will be enough to initiate bud burst which will be damaged by the returning freezing temperatures (Tolvanen, 1997). However, it will only be the buds formed during the previous year that will be damaged by freezing events, new bud formation will not be affected (Bokhorst *et al.*, 2008). Greater snow depths and short periods of winter warming will potentially cause another problem for *V. myrtillus*, since it begins to respire quickly in warmer temperatures (Billings & Mooney, 1968). Deeper snow may cause *V. myrtillus* to begin to use cryoprotective sugars for respiration, leaving it more susceptible to freezing damage or reduced growth next spring (it has been shown that this occurs to a degree under winter conditions without a warming period, as the carbohydrate content of *V. myrtillus* reduces over winter) (Ogren, 1996). *V. myrtillus* has also been shown to de-acclimate at temperatures of +10^{o}C in winter but not at +5^{o}C. This suggests that temperatures of +7^{o}C reached during winter warming will almost certainly cause *V. myrtillus* to de-acclimate partially. However if this occurs during mid-winter, the light conditions may reduce this risk slightly (Tolvanen, 1997). Cold acclimation is linked to circadian rhythms in plants (Fowler, Cook & Thomashow, 2005) and is therefore triggered by changes in both temperature and day length (Fowler & Thomashow, 2002). It is possible that as the day length will remain the same, de-acclimation may not be fully triggered by the temperature alone. Both early respiration and even partial de-acclimation by winter warming will significantly reduce the freezing tolerance of *V. myrtillus* when freezing temperatures return. If *V. myrtillus* becomes partially dehydrated during early snow melt, freezing tolerance will be maintained as dehydration limits the amount of respiration that can occur, therefore limiting the use of cryoprotective sugars for respiration (Ogren, 1996). However, dehydration itself causes delayed and stunted growth in the following season (Tahkokorpi et al., 2007). Despite being very susceptible to winter warming damage, *V. myrtillus* can recover quickly from spring and summer frosts, suggesting that it is always partially acclimated (Tolvanen, 1997).

In some studies *V. vitis-idaea* showed reduced growth and photosynthesis in response to simulated winter warming events causing snowmelt (Bokhorst *et al.*, 2008). In other studies where *V. vitis-idaea* overwintered without snow, plants showed desiccation stress due to light conditions in late winter causing water loss through transpiration but the ground remaining frozen so preventing water uptake through the roots (Taulavuori et al., 2011), suggesting climate change may also affect populations of *V. vitis-idaea*. However, it is thought that *V. vitis-idaea* can compensate for damage induced by winter warming events

with increased vegetative growth the following season (Bokhorst *et al.*, 2008). In a number of studies *V. uliginosum* showed no damage response to winter warming (Bokhorst *et al.*, 2011); indeed in a study carried out over 10 years in Abisko it was shown that winter warming events caused an increase in *V. uliginosum* abundance (Shaver & Jonasson, 1999).

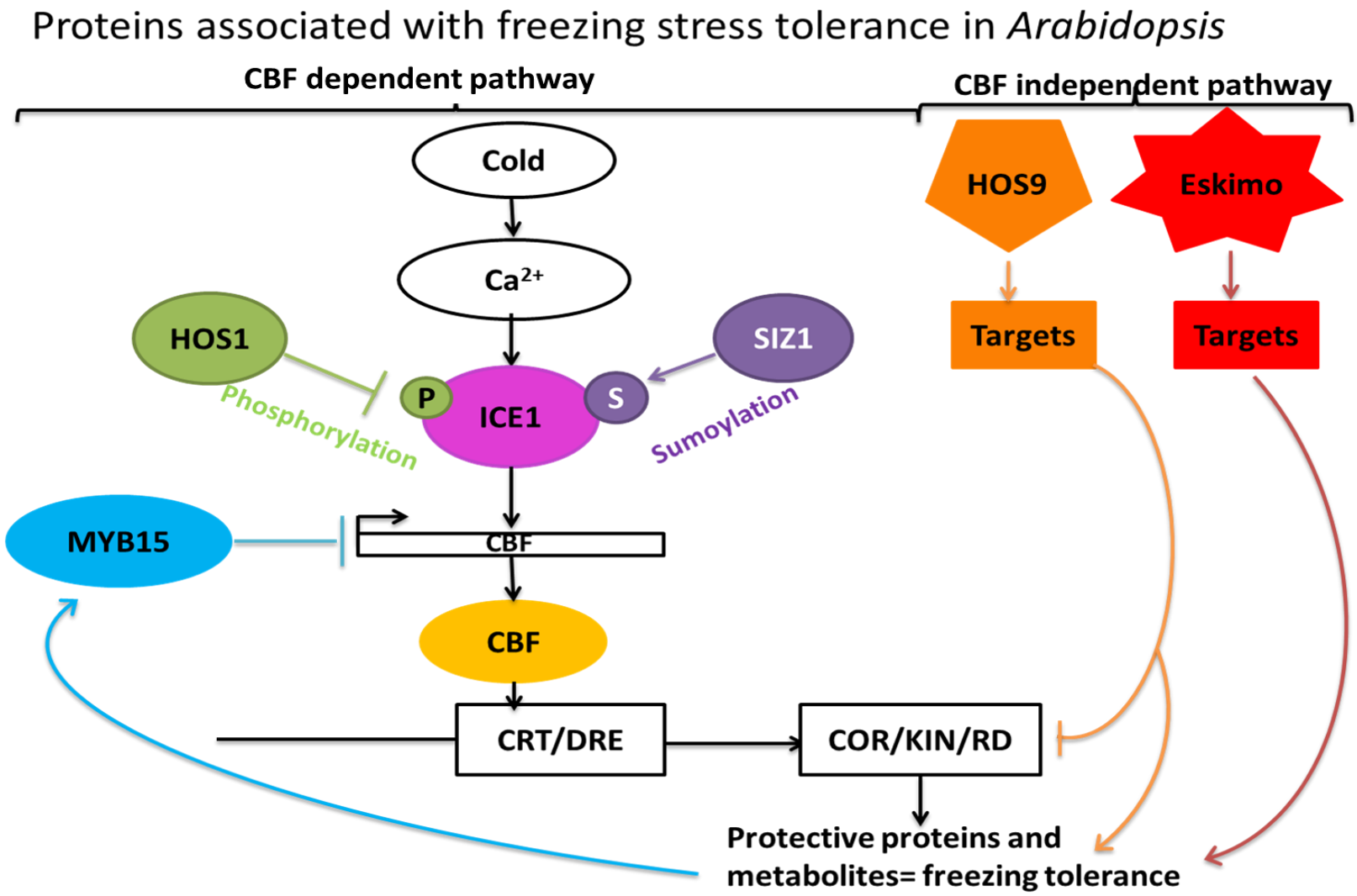

Figure 1.6: Components of the CBF/DREB1 dependent pathway and how they interact with each other in *Arabidopsis*, also independent proteins *ESK1* and *HOS9*

1.8 Cold sensing in *Arabidopsis*

The exact mechanism by which plants sense cold is not known but it is thought to be through a combination of two different processes. The first is through changes in protein conformation associated with the start of cold induced protein denaturation (Guy *et al.*, 1998); the second is through changes in membrane fluidity as the membranes begin to become more rigid in response to lower temperatures (Chinnusamy, Zhu & Zhu, 2006, Levitt, 1972). Cold stress signals include an increase in levels of cytosolic Ca^{2+} (Plieth et al., 1999, Tahtiharju et al., 1997); the plasma membrane becoming more rigid can cause a rearrangement of the actin cytoskeleton (which is an essential component of low temperature signalling), causing activation of Ca^{2+}. This occurs via an influx of cytosolic Ca^{2+} (Orvar et al., 1999), as well as release from stores inside the cell mediated by inositol triphosphate (IP_3) (Allen et al., 1995). IP_3 is thought to be upstream of *CBF/DREB1* expression in the CBF/DREB1 pathway (Catala et al., 2004, Chinnusamy *et al.*, 2006, Xiong et al., 2001). Calmodulins are one example of cold induced upstream positive regulators of *COR* gene expression via the CBF/DREB1 pathway (Townley & Knight, 2002). If Ca^{2+} channels in plant cells are blocked the plant becomes unable to cold acclimate; conversely, if high levels of Ca^{2+} are artificially induced inside the plant, cold acclimation specific gene induction is triggered even at ambient temperatures (Orvar *et al.*, 2000).

Abscisic acid (ABA) is a plant hormone produced as a stress signal in response to drought stress and can control stomatal closing in response to water loss, therefore preventing further dehydration (Mansfield T. A., 1995, Skriver & Mundy, 1990, Yamaguchi-Shinozaki & Shinozaki, 1994). As ABA signalling is thought to be specific to drought stress the CBF/DREB1 pathway is an ABA independent pathway. However, there is potentially cross talk between the two pathways at the transcriptional level (Shinozaki & Yamaguchi-Shinozaki, 2000) as freezing stress can lead to drought stress. ABA treated plants accumulate IP_3 (Xiong *et al.*, 2001) which plays an important role in cold acclimation, potentially through stress signals such as Ca^{2+} or H_2O_2 (Xiong *et al.*, 2001). ABA can induce an increase in expression of CBFs 1-3 which are usually cold induced (Knight et al., 2004), as well as inducing expression of ABA inducible *COR* genes (Gilmour & Thomashow, 1991). This was shown using mutants deficient in ABA, which were able to cold acclimate but not to the same level as plants with a functional ABA and CBF/DREB pathway, suggesting that both pathways are needed for effective cold acclimation (Gilmour & Thomashow, 1991).

The best characterised cold stress pathway is the CBF/DREB1 pathway (Figure 1.6). Each individual component of the CBF/DREB1 pathway is described in terms of function and interactions below.

1.8.1 *ICE1*

Inducer of *CBF* expression 1 (*ICE1*) is a transcription factor upstream of *CBF/DREB1* encoding a myelocytamatosis (MYC)-type helix loop helix (bHLH) transcription factor which activates expression of *CBF3* by binding to the MYC cis-element on the promoter of CBF3 in response to cold (Chinnusamy *et al.*, 2006). ICE1 is constitutively expressed and targeted to the nucleus but is only able to activate *CBF/DREB1* expression in cold conditions, suggesting that cold temperature induces post-translational modifications needed to produce a functional ICE1 activator (Chinnusamy et al., 2003). ICE1 may be involved in the regulation of *CBF1* and *2* (Van Buskirk & Thomashow, 2006, Zarka et al., 2003). *CBF/DREB1* genes contain a MYC recognition site (CANNTG) within the ICEr1 binding site (GGACACATGTCAGA) (Yamaguchi-Shinozaki & Shinozaki, 1994) for bHLH transcription factors such as ICE1 (Van Buskirk & Thomashow, 2006). Constitutively expressed ICE1 is potentially how *CBF/DREB1* can be expressed so quickly in response to cold as the activator transcription factor is already present. ICE1 is sumoylated by SIZ1 in response to cold, therefore inhibiting polyubiquitination of ICE1 by HOS1 allowing ICE1 to activate downstream *CBF/DREB1* expression (Miura et al., 2007). Un-sumoylated ICE1 induces the production of MYB15 which negatively regulates expression of *CBF/DREB1* (Miura *et al.*, 2007), whereas sumoylated ICE1 downregulates MYB15 expression, therefore allowing *CBF/DREB1* induction (Miura & Hasegawa, 2008). ICE is a multigene family in *Arabidopsis*; ICE2 is a regulator of *CBF1* and contains the same AP2 binding domain as ICE1 (Fursova et al., 2009). ICE1 can activate both *CBF/DREB1* dependent and independent genes (Chinnusamy *et al.*, 2006) as ABA can increase expression of *ICE1*; ICE1 may therefore regulate ABA mediated expression of the CBF/DREB1 pathway (Chinnusamy et al., 2003). Accumulation of salicylic acid (SA) reduces freezing tolerance and inhibits *CBF/DREB1* in *Arabidopsis*. Sumoylated ICE1 cold signalling is mediated by SA; ICE1 dependent signalling represses the accumulation of SA, therefore further preventing inhibition of CBF/DREB1 expression (Miura & Ohta, 2010). Overexpression of *ICE1* causes increased expression of *CBF/DREB1* and therefore increased freezing tolerance; mutants of *ICE* in *Arabidopsis* have impaired *CBF/DREB1* expression and therefore impaired freezing tolerance (Chinnusamy *et al.*, 2003).

1.8.2 *HOS1*

High expression of Osmotically Responsive gene 1 (*HOS1*) (Dong et al., 2006) is ubiquitously expressed across all tissues in *Arabidopsis* (Lee, Xiong, Gong, Ishitani, Stevenson & Zhu, 2001), and is involved in controlling cold acclimation, vernalisation (Lee *et al.*, 2001) and flowering time (Lazaro et al., 2012). *HOS1* encodes a protein similar to a ring finger domain protein that functions as an E3 ubiquitin ligase which targets specific regulatory proteins for degradation (Dong et al., 2006, Lee *et al.*, 2001). HOS1 is a negative regulator of *CBF/DREB1* (Ishitani et al., 1998, Lee et al., 2001) by targeting ICE1 for degradation. Unlike other E3 ligases, HOS1 can be active in both warm and cold conditions and doesn't show altered expression in either condition (Dong et al., 2006). Under cold conditions HOS1 is targeted to the nucleus, suggesting HOS1 may also play a role in cold signalling from the cytoplasm to the nucleus (Van Buskirk & Thomashow, 2006). However, *HOS1* is down regulated in response to cold, potentially allowing other cold signals to be amplified (Lee *et al.*, 2001). Over-expression of *HOS1* represses *CBF/DREB1* expression and causes increased freezing sensitivity due to high levels of HOS1 leading to reduced ICE1 even at ambient temperatures (Dong *et al.*, 2006).

1.8.3 *SIZ1*

Small ubiquitin-like modifier E3 ligase (SIZ1) accumulates under cold stress conditions (Lang, Mantyla, Welin, Sundberg & Palva, 1994, Miura *et al.*, 2007) and mediates the sumoylation of ICE1, thus increasing the activity of ICE1 and allowing induction of *CBF3* expression (Miura *et al.*, 2007). SIZ1 is thought to inhibit polyubiquitination of ICE1 which is induced by HOS1, therefore stabilising ICE1 in response to cold (Miura *et al.*, 2007). SIZ1 is constitutively expressed in ambient conditions, suggesting cold temperatures may induce post translational modifications to allow SIZ1 to alter ICE1 in response to cold (Miura et al., 2005). SIZ1 may also repress *MYB15* expression leading to activation of *CBF/DREB1* (Miura et al., 2007).

1.8.4 *MYB15*

There are three subfamilies of MYB proteins in *Arabidopsis* which are characterised by the number of MYB repeats present (Stracke et al., 2001). Each MYB binding domain comprises up to three repeats of a 53 amino acid helix turn helix motif with three tryptophan residues making up a MYB repeat sequence (Stracke et al., 2001). MYB transcription factors have highly conserved binding domains (Ding et al., 2009). Myelablastosis (MYB) transcription factor 15 (MYB15) is an R2R3 MYB (three MYB repeat) transcription factor expressed at constitutively low levels under ambient conditions; expression is then upregulated in

response to cold stress (Agarwal et al., 2006). It is thought that MYB15 interacts with ICE1 (Agarwal et al., 2006), although the signature sequences normally associated with MYB binding to helix loop helix protein interactions are not present (R2R3MYB to bHLH) (Zimmermann et al., 2004). MYB15 then goes on to bind to the MYB recognition sequence (TAACTG, (Urao et al., 1993) on the promoter of *CBF/DREB1* (Agarwal *et al.*, 2006). MYB15 to ICE binding prevents MYB15 induced inhibition of CBF/DREB1, therefore allowing CBF/DREB1 expression under cold conditions. Overexpression of MYB15 in *Arabidopsis* reduces freezing tolerance (Agarwal et al., 2006) but causes plants to become hypersensitive to ABA, thus improving drought tolerance by inducing changes such as stomatal closure (Ding et al., 2009). *MYB15* mutant plants display increased freezing tolerance (Agarwal et al., 2006). As *MYB15* is a member of a large family of R2R3 MYB transcription factors, it is possible that other members of the MYB family are also involved with the CBF/DREB1 signalling pathway (Agarwal et al., 2006).

1.8.5 *CBF/DREB1* in *Arabidopsis*

CBF/DREB1 expression in response to cold is induced by ICE binding and goes on to activate expression of cold on-regulated genes (*COR genes*) (Gilmour et al., 1998). C-repeat binding factors (CBFs) are one of the most characterised groups of transcription factors associated with cold acclimation and freezing tolerance in plants, also known as the dehydration responsive element binding protein 1 (DREB1s) (Liu et al., 1998, Shinwari et al., 1998). Cold regulatory elements were identified on each *COR* gene as a site of regulation (Yamaguchi-Shinozaki & Shinozaki, 1994) which is a 9 bp recognition sequence (TACCGACAT) known as a dehydration responsive element (DRE); the CCGAC sequence in the middle is called the C-repeat element (CRT) (Baker et al., 1994). CBF/DREB1 proteins bind the CRT/DRE recognition sequence in *COR* gene promoters (Stockinger et al., 1997) in response to cold temperatures as part of cold acclimation (Sarhan & Danyluk, 1998). CBF/DREB1 is a small family of three closely related transcription factors that are all cold induced (CBF1-3) (Gilmour et al., 1998, Shinwari et al., 1998). In *Arabidopsis* there is a fourth *CBF/DREB1 (CBF4)* which is solely drought responsive (Haake, et al., 2002). All three cold induced *CBF/DREB1s* are low copy number genes (Stockinger et al., 1997) which encode production of proteins that are approximately 24kDa in size and contain an AP2 region (Riechmann & Meyerowitz, 1998) which contains a DNA binding domain (Ohmetakagi & Shinshi, 1995).

CBF/DREB1 transcript levels increase after 15minutes of exposure to cold temperatures in *Arabidopsis* (Gilmour *et al.*, 1998, Liu *et al.*, 1998) and activate up to 12% of *COR* genes (Vogel et al., 2006). *CBF/DREB1* transcript reaches a maximum 2h after cold is initially

sensed (Gilmour et al., 1998, Liu et al., 1998). It is suggested that CBF1 and CBF3 work together as a pair and CBF2 works independently on a different sub-set of *COR* genes. This hypothesis also states that there are two different groups of *COR* genes; one group of *COR* genes needs both CBF1 and CBF3 to be expressed simultaneously to trigger activation, whereas the second group only needs either CBF1 or CBF3 to be active (Novillo et al., 2007). However, constitutively expressing *CBF1* in *Arabidopsis* has been shown to cause overexpression of the *COR* genes controlled by the CRT/DRE element even in the absence of cold (Jaglo-Ottosen et al., 1998). Over-expression of all three *CBF/DREB1s* individually has been shown to induce the same freezing tolerance in the absence of cold (Jaglo-Ottosen et al., 1998, Liu et al., 1998, Shinwari et al., 1998), showing that each *CBF/DREB1* must be able to activate the same range of *COR* genes independently.

The CBF/DREB1 pathway is highly conserved between plant species (Jiang, Iu & Singh, 1996, Thomashow et al., 1990) as are the AP2 binding domains (Weigel, 1995). The CBF/DREB1 pathway being well characterised across a number of species, it is a good potential target for crop improvement. Furthermore, by modifying or inserting a transcription factor it is possible to activate a whole range of genes involved in acquiring freezing tolerance (Sarhan & Danyluk, 1998).

1.8.6 *COR* genes

Guy et al. (1985) showed that changes in gene expression lead to cold acclimation. Cold acclimation is caused by the expression of a large number of genes known as cold regulated genes (*COR* genes), each of which plays a small additive role to give a combined result of freezing tolerance (Guy et al., 1985). A large number of cold on regulated genes (*COR genes*) has been identified in *Arabidopsis;* these genes have been cloned on the basis of their induction and expression at low temperatures (Hughes & Dunn, 1996). Some *COR* genes encode polypeptides that are hydrophilic and remain soluble when boiling in aqueous solutions (boiling stable) (Hajela et al., 1990). Other *COR* genes encode a number of proteins and processes such as: heat shock proteins (HSP) which stabilise proteins from freezing induced denaturation (Krishna et al., 1995); fatty acid desaturases to aid alterations in lipid composition (Gibson et al., 1994); signalling pathway proteins such as calmodulins (Polisensky & Braam, 1996); and a large number of hydrophilic cryoprotective proteins to prevent membrane desiccation (Thomashow et al., 1990). Expression of a number of *COR* genes has been found to be regulated by both cold and drought stress (Hajela et al., 1990, Liu et al., 1998); mRNAs from *COR* genes accumulate two hours after cold is sensed (Gilmour et al., 1998, Liu et al., 1998) and remain until the plant returns to

ambient conditions (Hughes & Dunn, 1996). Promoter regions of *COR* genes contain cis-elements such as CRT/DRE elements, myelablastosis (MYB) or myelocytamatosis (MYC) elements or ABA responsive elements (ABRE) (Yamaguchi-Shinozaki & Shinozaki, 1994). The CRT/DRE element is present in the promoter regions of many *COR* genes including *COR15A*, *COR78* (*LTI78*- Low temperature inducible 78) and *COR6.6* (Baker et al., 1994, Nordin et al., 1993).

A well characterised family of *COR* genes is the cold inducible family (*KIN*), members of which are expressed in response to low temperature or drought (Kurkela & Borgfranck, 1992). *KIN1* (also known as *COR 6.6)* produces alanine rich proteins that have homology to the antifreeze proteins produced in Arctic flounder (Gilmour et al., 1992, Kurkela & Franck, 1990). Both *KIN1* and *KIN2* are similar and produce similar antifreeze proteins, both showing an expression level much increased from the basal expression level in response to cold; *KIN2* expression also increases in response to drought and salinity stress (Kurkela & Borgfranck, 1992).

Another well characterised *COR* gene is *COR15A* which is also expressed in response to both cold and drought stress in *Arabidopsis* (Hajela et al., 1990). *COR15A* encodes a hydrophilic cryoprotective protein which is boiling stable, and potentially counteracts the effect of L-lactate dehydrogenase enzymes (Lin & Thomashow, 1992). Constitutive expression of *COR15A* in *Arabidopsis* increases freezing tolerance in both the chloroplasts and protoplasts, suggesting that COR15A stabilises membranes against freeze induced damage (Artus et al., 1996, Thomashow et al., 1996).

1.8.7 Conservation of the CBF/DREB1 pathway in other species

Since the CBF/DREB1 pathway has been characterised and shown to be important in freezing tolerance in *Arabidopsis*, a number of studies have been carried out to look at the function of *CBF/DREB1* homologues in crop species. CBF/DREB1 has been isolated from a number of crop species including, *Triticum aestivum* (wheat), *Secale cereal* (rye), *Hordeum vulgare* (barley), *Oryza sativa* (rice), *Sorghum bicolor* (sorghum) and *Pennisetum glaucum* (millet), as well as a number of chilling sensitive crops such as *Cocos nucifera* (coconut).

Both *ICE* and *CBF/DREB1* genes have been isolated from *Camellia sinensis* (tea) *csCBF1*, *csICE1* (Wang et al., 2012). Homologues of *ICE1* have also been found in various species, including mdclbHLH1 from *Malus x domestica* (apple) (Feng et al., 2012); *EcaICE1* from *Eucalyptus camaldulensis* (Eucalyptus) (Lin et al., 2014); also *OSICE1* and *OSICE2* from *O. sativa* which were associated with the induction of trehalose synthesis (Nakamura et al.,

2011). *TaICE41* and *TaICE87* from *T. aestivum* have been shown to induce increased freezing tolerance when overexpressed in *Arabidopsis* (Badawi et al., 2008).

In a number of freezing tolerant and freezing sensitive species CBF/DREB1 pathways have been found that have a high level of conservation, with large sections of the protein sequence, in particular the COOH end region and the *COR* gene binding domain, conserved. Overexpression of *ICE1* from *Arabidopsis* in *Cucumis sativus* (cucumber) increased freezing tolerance (Liu et al., 2010); this would not have been possible if there was not a high level of conservation between species.

There are also a number of variations that have being found in the sequence and way the CBF/DREB1 pathway functions in other species. *L. esculentum*, despite being chilling sensitive, has three homologues of *CBF/DREB1* (*LeCBF1-3*). *LeCBF1* was found to be cold inducible but could not induce chilling tolerance even when overexpressed in tomato. Over expression of *Arabidopsis CBF1* in tomato could not induce chilling tolerance in tomato but overexpression of *LeCBF* in *Arabidopsis* induced freezing tolerance, suggesting that different genes are induced by CBF/DREB1 in tomato and *Arabidopsis* (Zhang et al., 2004). *Vitis vinifera spp*. (grape) was also shown to have a different *CBF/DREB1* pathway to that in *Arabidopsis* (Xiao et al., 2006).

1.9 CBF/DREB1 in *Vaccinium*

CBF/DREB1 sequences for two species of *Vaccinium* (*V. vitis-idaea* and *V. corymbosum*) had been published prior to this project starting. A project to sequence the genome of *V. corymbosum* (American blueberry) and *V. macrocarpon* (cranberry) is still on going because of the commercial importance of *V. corymbosum* and *V. macrocarpon* in America (Die & Rowland, 2013, Polashock et al., 2014, Rowland et al., 2012). Improved freezing tolerance to survive winter and spring frosts is a desired trait as many blueberry cultivars are freezing sensitive (Moore, 1993). It is thought that different cultivars of blueberry exhibit varying levels of deacclimation in response to winter warming due to differences in genotype (Arora et al., 2004).

CBF/DREB1 (VcDREB1) isolated from *V. corymbosum* induced freezing tolerance when over-expressed in *Arabidopsis,* but not the same level of freezing tolerance seen in cold acclimated wt plants. CBF/DREB1 from *V. corymbosum* induced expression of *KIN2* and *LTI78,* but not *COR414* or *COR15A,* in *Arabidopsis* (Polashock et al., 2010). A project to assemble ESTs for *V. corymbosum* during processes such as cold acclimation, flowering and fruit production has been conducted (Rowland et al., 2012). A number of dehydrin proteins thought to be associated with cold acclimation has been isolated from *V. corymbosum*;

isolated after 300h exposure to cold, their levels decreased again after bud break (Arora et al., 2004, Muthalif & Rowland, 1994).

The *CBF/DREB1* sequence for a Chinese population of *Vaccinium vitis-idaea* has also been sequenced (*VviDREB1*); this has a 35 amino acid extension at the N-terminal end when compared to the CBF/DREB1 sequence in *Arabidopsis*. The *CBF/DREB1* from *V. vitis-idaea* was found to be targeted to the nucleus when bombarded into epidermal cells of onion (*Allium cepa*) and expression of the gene is regulated by ABA as well as by cold (Wang et al., 2010).

1.10 *CBF/DREB1* independent pathways associated with freezing tolerance in *Arabidopsis*

1.10.1 Eskimo

Eskimo (*ESK1*) encodes a 57kDa protein in *Arabidopsis* which is a member of the DUF231 domain protein family; there are 45 proteins in this family, all of unknown function (Xin et al., 2007). ESK1 is thought to be a regulatory component of cold acclimation (Xin & Browse, 1998) and possibly involved with water homeostasis (Bouchabke-Coussa et al., 2008, Lugan et al., 2009). *ESK1* mutants exhibited an increase in freezing tolerance in *Arabidopsis* of 5.5^{o}C without a period of acclimation (Xin et al., 2007) and were found to show high levels of proline without any *COR* gene expression (Xin & Browse, 1998). This suggests that *ESK1* is in a separate pathway from *CBF/DREB1* and *HOS9* and that in wt *Arabidopsis* ESK1 functions as a positive regulator of proline biosynthesis (Xin & Browse, 1998). Under ambient conditions, pyrroline-5-carboxylate synthase (P5CS) negatively regulates the production of proline derived glutamate biosynthesis (Strizhov et al., 1997). ESK1 negatively regulates P5CS expression allowing upregulation of proline biosynthesis (Xin & Browse, 1998). *ESK1* can induce expression of genes that are also expressed in response to salt, osmotic and ABA stress signals (Xin et al., 2007). *ESK1* mutants shown an altered basic water content and a lower transpiration rate than wt *Arabidopsis* (Lugan et al., 2009); these would lead to increased physiological freezing tolerance without altering cold signalling pathways.

1.10.2 HOS9

High expression of Osmotically responsive genes 9 (*HOS9*) and also 10 (*HOS10*) are constitutively expressed homeodomain transcription factors similar to WUSCHEL (WUS), pressed flower (PRS) and an R2R3 type MYB transcription factor that is localised to the nucleus (Zhu et al., 2004). *HOS9* controls ~175 genes, of which 49 are cold induced and

none of which is controlled by *CBF/DREB1* (Xiong et al., 2002). HOS9 is thought to play a role in freezing tolerance, although it is not known whether it is an entirely CBF/DREB1 independent pathway or whether HOS9 plays a role in the CBF/DREB1 pathway downstream of *CBF/DREB1* (Van Buskirk & Thomashow, 2006). HOS9 is also associated with growth rate, flowering rate and flowering time (Xiong et al., 2002).

1.11 Overall aims of the project

As climate change is causing increasingly unpredictable weather, finding new ways of improving crops is becoming more of a priority. Many studies have focussed on CBF/DREB1s from chilling sensitive crops to establish whether this is the basis of why they are not cold tolerant. Some studies have investigated whether adding components from the CBF/DREB1 pathway in *Arabidopsis* can improve either cold or freezing tolerance in crop species. However, there is comparatively less research performed at a molecular level on highly freezing tolerant species to determine why they are so freezing tolerant. Therefore, the main aim of the project described in this thesis was to establish whether species of *Vaccinium* collected in the sub-Arctic, use the CBF/DREB1 pathway as part of cold acclimation; and if so, does CBF/DREB1 work in the same way as in *Arabidopsis*? *Vaccinium* spp. found in the Arctic have to be able to cold acclimate efficiently; and to a possibly higher level of freezing tolerance than *Arabidopsis* in order to survive. Results from *Vaccinium* could therefore either further highlight the importance of the CBF/DREB1 pathway; or potentially highlight more important alternative pathways found in freezing tolerant species. To tackle these issues, research was split into the specific questions detailed below.

Are there differences in the protein sequence of CBF/DREB1s from each of the *Vaccinium* species? *(Chapter 3)*

The first step in answering this question was to identify clone and sequence *CBF/DREB1* DNA coding regions from each of the three species of sub-Arctic *Vaccinium*. Following this; the aim was to establish whether there were differences in the deduced CBF/DREB1 protein sequence between each of the three species of *Vaccinium*. The sequences could then be compared; to identify any regions of the *CBF/DREB1* sequences that were conserved between *Vaccinium* and *Arabidopsis.* As any such differences found between the *CBF/DREB1*sequences from *Arabidopsis* and *Vaccinium,* may account for the differences in freezing tolerance, and therefore winter survival strategy between *Vaccinium* and *Arabidopsis*.

Are *Vaccinium CBF/DREB1s* functional in *Arabidopsis*? *(Chapter 3)*

The obtained *CBF/DREB1* coding sequences were then used to create overexpression vectors to test the function of *Vaccinium CBF/DREB1* genes in *Arabidopsis*. This was to

establish whether there are differences in potency between the three *Vaccinium* CBF/DREB1s when expressed in the same genetic background.
The transgenic *Arabidopsis* lines produced were characterised in terms of altered freezing tolerance, changes in *COR* gene expression and general growth phenotype. This highlighted differences in growth and freezing tolerance induced by the respective *Vaccinium CBF/DREB1* genes. It also demonstrated whether overexpression of the *Vaccinium* CBF/DREB1s could induce freezing tolerance in *Arabidopsis* showing conservation between the *Arabidopsis* and *Vaccinium* CBF/DREB1 pathways.

Why does only the CBF/DREB1 from *V. myrtillus* induce freezing tolerance when overexpressed in *Arabidopsis?* *(Chapter 4)*
Chapter 4 describes investigating the reasons for the altered phenotype; and increased freezing tolerance induced by the *Vaccinium CBF/DREB1s* overexpressed in *Arabidopsis*. Additionally the aim was to determine why there was a difference in induced freezing tolerance and phenotype when *CBF/DREB1* genes from different species of *Vaccinium* were used.

Are the three *Vaccinium* CBF/DREB1s targeted to different parts of the cell accounting for their different activities? *(Chapter 4)*
GFP-CBF/DREB1 fusion overexpression constructs were produced for each of the three *Vaccinium CBF/DREB1s* and expressed transiently in *Nicotiana benthamiana.* These were imaged, to test whether the differences in freezing tolerance induced by the three *Vaccinium CBF/DREB1s* was due to a difference in protein localisation.

Are there differences in protein stability between the *Vaccinium* CBF/DREB1s when transiently expressed? *(Chapter 4)*
Extracted protein from the infiltrated *N. benthamiana* leaves was used for analysis by Western blot, to determine whether the differences in freezing tolerance were caused by an altered level of protein between the three *Vaccinium* CBF/DREB1 proteins. As the expression constructs were identical in every way other than the coding regions themselves, any differences in protein levels are likely to reflect differences in protein stability in *N. benthamiana*.

Are the differences in freezing tolerance induced by the *Vaccinium* CBF/DREB1s due to differing abilities to induce *COR* gene expression? *(Chapter 4)*

The ability of each CBF/DREB1 to bind and activate *COR* genes in *N. benthamiana* was tested; using reporter gene assays. To establish whether the small number of differences in amino acid sequence between the CBF/DREB1 from the three *Vaccinium* species; altered their ability to bind and activate *COR* genes in *N. benthamiana*.

Could the differences in *COR* gene binding and activation for *V. uliginosum* and *V. vitis-idaea* be improved by systematic mutations to change differing amino acids to match *V. myrtillus*? *(Chapter 4)*

As differences in stability and activation of CRT/DRE between the CBF/DREB1s from each species were demonstrated, these differences had to be due to the differences in amino acid sequence between the three species. Amino acids that differed between the three species within the *COR* gene binding domain; were highlighted and used as targets for site directed mutagenesis. Five target sites were systematically altered to match the amino acid sequence of the CBF/DREB1 from *V. myrtillus* (one mutation per construct). This included a possible ubiquitination site, which was unique to *V. uliginosum* to establish whether the mutagenesis could improve stability and therefore *COR* gene binding. The mutant *Vaccinium CBF/DREB1* constructs were then used to repeat the reporter gene assays; using wt *Vaccinium* CBF/DREB1 constructs as a control. This established whether the mutations improved the ability of each CBF/DREB1 to bind and activate *COR gene* expression.

Which proteins interact with CBF/DREB1 from both *Vaccinium* and *Arabidopsis*? *(Chapter 5)*

The work carried out in Chapter 5 was carried out with the aim of establishing which proteins/DNA interact with CBF/DREB1 from both *Vaccinium* and *Arabidopsis*. Strep tagged lines for *Arabidopsis* CBF/DREB1 were produced and tagged proteins attempted to be isolated using strep column pull downs. As this was unsuccessful, GFP-CBF/DREB1 fusion constructs were made for the three *Vaccinium* CBF/DREB1s and *Arabidopsis* CBF/DREB1. These constructs were then transformed into *Arabidopsis* to produce stable transgenic lines for each. The *Vaccinium* constructs made by site directed mutagenesis, were also used to produce GFP-CBF/DREB1 fusions. These fusion constructs were then used to carry out GFP pull downs, from transient expressions in *N. benthamiana*. GFP pull downs were also

carried out using *Arabidopsis* tissue from the *V. uliginosum* GFP-CBF/DREB1 stable transgenic lines.

Are there post-translational modifications of CBF/DREB1? *(Chapter 5)*

Mass spectroscopy was used to analyse any proteins successfully isolated from the pull down experiments, to identify the CBF/DREB1 and any protein interactors that may have been 'pulled down' along with them. The transiently expressed, site directed mutagenesis constructs were used to try and identifying any differences between the interactions of the wt *Vaccinium* CBF/DREB1 proteins and the mutated *Vaccinium* CBF/DREB1 proteins.

What are the expression patterns of *CBF/DREB1* in *Vaccinium*? *(Chapter 6)*

An ongoing experiment carried out over three years was to measure *CBF/DREB1* expression in the three species of Arctic *Vaccinium* in response to cold. This experiment was ongoing as it required carrying out experiments on plants from Abisko around midsummer when the plants were least likely to be cold acclimated. This left a small window of time each summer for cold treatment experiments to be carried out. The results of the preliminary cold treatment experiments showed that the experiment needed to be extended into a time course, instead of a single time point. This was to try and get a full picture of what the *CBF/DREB1* response of each of the three *Vaccinium* species was over a 72h window in response to cold.

Are there differences in expression of other components of the CBF/DREB1 pathway and non-CBF/DREB1 pathways between the three *Vaccinium* species? *(Chapter 6)*

To create a more detailed cold induced gene expression pattern for each of the three species, partial sequences of other components of the CBF/DREB1 pathway were isolated and sequenced as well as two CBF/DREB1 pathway independent genes. These sequences were then used to design qPCR primers to test the expression of each gene in *Vaccinium* samples. A larger scale time course experiment was carried out in Umeå with samples harvested for each of the three species over a period of 72h; to investigate their response to a cold treatment. qPCR was then used to establish an expression pattern for *CBF/DREB1* for each of the three species over the 72h period as well as expression patterns for *ICE2*; *MYB15*; *SIZ1* ;and *ESK1;* to build up a more complete picture of the response of each species to cold.

2 Materials and methods

2.1 Materials

2.1.1 **Chemicals**

All chemicals and media used were supplied by one of the following companies unless stated otherwise:

BDH Laboratory Supplies Ltd, (Lutterworth, Leicestershire, UK)

Melford laboratories Ltd, (Chelasworth, Ipswich, Suffolk, UK)

Fisher Scientific UK ltd, (Loughborough, Leicestershire, UK)

Bioline (London, UK)

Sigma-Aldrich Ltd, (Dorset, UK)

MERK chemicals Ltd, (Nottingham, UK)

2.1.2 **Plant materials**

Arabidopsis thaliana (*A. thaliana*) ecotype Columbia (Col-0) from Lehle seeds (Round rock, Texas, USA.)

35S::CBF2-C-Strep, 35S::CBF2-N-Strep, CBF2-N-strep and CBF2-C-Strep *Arabidopsis* seeds obtained from Alex Sargeant (Knight lab)

Vaccinium myrtillus, *Vaccinium uliginosum* and *Vaccinium vitis-idaea* samples were collected in Abisko, Sweden (68^{o}20'N)

Seeds for *Nicotiana benthamiana* (*N. Benthamiana*) were a kind gift from Dr Tim Hawkins (Durham University, UK).

2.1.3 **Bacterial material**

Escherichia coli (*E. coli*) strain DH5α (Bernard & Couturier, 1992) was obtained from Invitrogen Ltd. (Paisley, Renfrewshire, UK).

Agrobacterium tumefaciens C58C1 (Deblaere et al., 1985) were generated by Dr Piers Helmsly.

2.1.4 **Modifying enzymes**

All DNA and RNA modifying enzymes were purchased from Bioline, Invitrogen, Fisher, (Applied Biosystems, (AB) California, USA), Qiagen, (Sussex, UK), Promega, (Wisconsin, USA) Primer Design Ltd. (Southampton, UK) or New England Biolabs Ltd. (NEB) (Hitchin, Hertfordshire, UK).

2.1.5 Nucleotides

All nucleotides were obtained from Invitrogen via Thermo Fisher.

2.1.6 Sterilisation

2.1.7 Solution sterilisation

All growth media and nucleotide extraction buffers were sterilised by autoclaving at 121^{o}C and 10^5 Pa for 20 minutes. (RNA extraction buffers were treated with 0.1% DEPC (v/v) at 37^{o}C overnight prior to autoclaving).

2.1.8 Seed Sterilisation

2.1.8.1 Ethanol surface sterilisation

Arabidopsis seeds sterilised with 70% (v/v) ethanol in 1.5ml microtubes for 5 minutes, transferred to sterile filter paper (Whatman International Ltd, Maidstone, Kent, UK) to air dry in a sterile laminar flow hood.

2.1.8.2 Bleach surface sterilisation

Seeds collected from *A. tumefaciens* dipped plants were first ethanol sterilised (as above). Then shaken in a solution of 10% (v/v) sodium hypochlorite, 0.25% (w/v) sodium dodecyl sulphate (SDS) and autoclaved milliQ water for 10 minutes. The seeds were then washed six times with sterile water (10 minutes per wash) before being spread onto solid agar germination medium and left to dry in a sterile laminar flow hood.

2.2 Methods

2.3 Growth media

2.3.1 Plant growth media

Sterilised *Arabidopsis* seeds were grown on solid MS (Murashagie and Skoog) plates (Murashige & Skoog, 1962). Comprising of plant tissue culture grade agar (Sigma-Aldrich) 0.8% (w/v) and 1x Murashige and Skoog salts including vitamins (MS: Duchefa Biochemie BV, Harlem, Netherlands). The pH was adjusted to 5.8 by adding 0.1M KOH before autoclaving.

For seeds collected from *A. tumefaciens* dipped plants appropriate antibiotics were added to the growth medium when the temperature had cooled to below 50^{o}C after autoclaving.

Seeds collected from dipped plants for BASTA selection were grown on trays of pearlite (Arthur J. Bowers) with a top layer of sand, seeds sprinkled onto the top sand layer. Watered with ¼ strength (w/v) MS media.

Seeds for 35S::*Vaccinium* CBF constructs were grown on MS plates containing 50µm Gibberellic acid, (GA) dissolved as a 10mM stock solution in methanol before diluting to the final concentration in the growth media.

N. benthamiana seeds were sprinkled onto pots of compost. Seedlings transferred to bigger separate pots as required.

Seedlings grown to maturity by carefully transferring them to re-hydrated peat plugs, (Jiffy products international AS, Norway) after 7 days of growth on MS. (For some constructs and GA plates plants were transferred when they reached the same size as 7 day old wt *Arabidopsis*). Individual plants were grown on 38mm (diameter) peat plugs whereas, 42mm peat plugs were used for 3 seedlings to use for *A. tumefaciens* dipping.

2.3.2 Bacterial growth media

E. coli and *A. tumefaciens* were grown on solid agar plates (with the appropriate antibiotics) comprising of 1.5% (w/v) micro agar (Sigma-Aldrich) and 20% (w/v) Luria-Bertani (LB) medium (Sigma-Aldrich). Overnight cultures were made from 20% (w/v) LB medium (all autoclaved before use).

2.3.3 Antibiotics

All antibiotics were bought from Melford laboratories Ltd, (Lutterworth, Leicestershire, UK). Antibiotics were added (as required) to autoclaved MS or LB once it had cooled below 50^{o}C. Concentrations used are listed in Table .2.1

Table .2.1: Concentrations of both working and stock solution antibiotics used for both bacterial and plant culture plates.

Concentrations of antibiotics added to LB and MS media.				
	Antibiotic	**Working concentration (µg/ml)**	**Stock concentration (mg/ml)**	**Stock solution made up in:**
Plant	Kanamycin	50	100	water
	Phosphinothricin (Basta)	10	10	water
	Timentin	200	200	water
Bacteria	Ampicillin	100	100	water
	Kanamycin	100	100	water
	Spectinomycin	100	50	water
	Rifampicin	100	50	DMSO

2.4 Growth conditions

2.4.1 Plant growth conditions

All seeds sewn on either Basta trays or MS plates were stratified at 5^{o}C for a minimum of 48h in darkness to ensure uniform germination and growth. Plates were then transferred to a percival (CU-36L5D, CLF plant climatics, Emersacker, Germany) at a temperature of 20^{o}C and 16/8h photoperiod with a light intensity of 150 µmol $m^{-2}s^{-1}$ until being transferred to peat plugs after 7 days.

Mature plants on peat plugs were grown in growth chambers at a temperature of 20^{o}C and 16/8h photoperiod with a light intensity of 150 µmol $m^{-2}s^{-1}$. Seeds were collected using the Aracon system (Beta Tech, Ghent, Belgium) to contain each plant. Compost was watered with 100mg $litre^{-1}$intercept (Everris International, Ipswich, UK) and Baby bio (Bayer Garden, UK) following manufactures instructions weekly until the siliques had developed and plant were ready to senesce and dry out.

Seedlings on Basta trays were grown until they were clear transformants (see2.14.2.2) then transferred to peat plugs and treated in the same way as above.

2.4.2 Bacterial growth conditions

E. coli were incubated at 37°C on static LB plates or shaking LB overnight cultures (200rpm). *A. tumefaciens* was incubated at 28°C on static plates for 48h or overnight in liquid LB cultures shaken at 200rpm.

2.5 Plant stress treatments

2.5.1 *Vaccinium* stress treatments

For details of *Vaccinium* stress treatments please see Chapter 6.

2.6 *Arabidopsis* stress treatments

2.6.1 Freezing assays

Transgenic *Arabidopsis* lines overexpressing vCBF were tested for *vCBF* and *COR* gene expression in a number of independent lines for each construct. A high and low *vCBF*-expressing line was chosen for each of the 3 species. Seeds from each of the 6 chosen lines and Col-0 wild type control were germinated and grown for 5 weeks (in the same way as described above) then transferred straight to -7°C for 24h in a Sanyo MIR 254 incubator in darkness. The plants were then left to thaw and returned to their original growth conditions. Before being transferred to the freezing chamber each plant was individually photographed and again 3 days after freezing. (See 2.15.3)

2.7 Nucleic acid extraction

2.7.1 Plant genomic DNA extraction

2.7.2 *Vaccinium*

2.7.2.1 *CTAB DNA extraction*

Using an adapted method from (Doyle & Doyle, 1987, Strange, 1998).

Around 100mg of *Vaccinium* leaf tissue was ground in a pre-chilled pestle and mortar. 800µl of extraction buffer (Appendix A -Solutions and Media recipes) which had been pre heated to 60°C was added to the ground leaves. Once defrosted the tissue and buffer was transferred to a 1.5ml microtube and 3µl of β-mercaptoethanol was added to each tube, mixed by inversion and incubated at 60°C for 30 minutes. 500µl of chloroform isoamyl-alcohol (24:1) and added to each tube and mixed by inversion. Centrifuged at 13000rpm for 5 minutes and the upper aqueous layer transferred to a new microtube, this set was

repeated until the upper aqueous layer was no longer cloudy. (However there was sometimes some colour, greenish brown). 500µl of ice cold isopropanol was added to each sample, mixed by inversion and incubated at -20^{o}C for 30 minutes. The tubes were then centrifuged at 13000rpm (Genfuge 24D, Progen, G H Zeal Ltd, London UK) for 10 minutes after which the supernatant was removed and discarded. 800µl of 70% ethanol (v/v) was added to each tube and mixed by pipetting to wash the pellet then incubated at room temperature for 20 minutes. The samples were then centrifuged at 13000rpm for 5 minutes and again the supernatant was removed and discarded. The tubes were then put into a hot block, (AccuBlock digital dry bath, Labet International Inc, New Jersey, USA) at 37^{o}C with the lids open to allow the pellet to dry (for around 30 minutes). All samples were then resuspended in 100µl of TE buffer (Appendix A -Solutions and Media recipes) and RNAse (Sigma-Aldrich Ltd) at a final concentration of 10µg/µl and incubated at 37^{o}C for 30 minutes. 500µl of ice cold isopropanol was added to each sample and mixed by inversion. DNA was precipitated at -20^{o}C for 30 minutes. Samples were then centrifuged at 13000rpm for 10 minutes and the supernatant was removed and discarded. 800µL of 70% ethanol (v/v) were added, mixed and incubated for 20 minutes at room temperature before being spun down at 13000rpm for 5 minutes. The supernatant was removed and discarded and the pellet was again left to air dry at 37^{o}C then resuspended in 100µl of TE buffer and left to dissolve fully overnight at 4^{o}C

2.7.2.2 ***Phenol-chloroform DNA clean up***

A further clean up step was required for the DNA extracted from *V. vitis-idaea.* 25µl of extracted DNA was further diluted to a total volume 400µl in TE buffer. 400µl of phenol-chloroform was then added to each sample and mixed to an emulsion by vortexing. The samples were then spun down for 5 minutes at 13000rpm and the upper aqueous layer was transferred to a new microtube. This phenol-chloroform step was repeated until no precipitate was formed between the aqueous and chloroform layers. 1/10th of the volume (of the aqueous layer) of 3M sodium acetate at pH 5.2 and two volumes of 100% ethanol was added to each sample, mixed by vortexing and left to precipitate at -80^{o}C for 30 minutes. The samples were then centrifuged at 16000g for 10 minutes and the supernatant was removed and discarded. The pellets were washed by adding 1ml of 80% ethanol (v/v) and centrifuged at 13000rpm in a microfuge for 5 minutes. The supernatant again was removed and discarded and the pellet was left to air dry. Samples were then resuspended in the same 25µl volume of TE buffer.

2.7.3 *Arabidopsis* DNA extraction

2.7.3.1 *Edwards Prep DNA extraction*

Adapted from Edwards et al., (1991).

Around 6-7 day old seedlings (or one leaf from a 2-3 week old plant) were collected from MS agar plates carefully and placed into a 1.5ml microtube. Each sample was flash frozen in liquid nitrogen then carefully removed from liquid nitrogen and ground using a micropestle. 400µl of Edwards extraction buffer (Appendix A -Solutions and Media recipes) was added to each sample halfway through grinding. Samples were vortexed to mix and then spun down in a microfuge for 1 minute at full speed (~16000g). The supernatant was transferred to a new microtube and 300µl of isopropanol was added and mixed by inverting then incubated at room temperature for 2 minutes. The samples were then spun down in a microfuge at full speed for 10 minutes and the supernatant was removed and discarded. The pellets were then dried using a vacuum concentrator (5031 Eppendorf UK Ltd. Stevenage, UK) to remove any remaining supernatant before resuspending in TE buffer. Samples were incubated at 4^{o}C overnight to allow the DNA to dissolve.

2.7.4 Bacterial plasmid DNA purification

A Wizard ® plus SV miniprep kit (Promega) was used to extract bacterial plasmid DNA according to manufacturer's instructions. Samples were processed according to instructions, bacterial overnight liquid cultures were spun down and the bacteria pellet resuspended. The bacteria were lysed then treated with alkaline phosphatase before precipitating cell debris and removing it from the suspension by centrifuging. The plasmid DNA was then bound to a column and washed with buffers and ethanol before eluting in nuclease free water.

2.7.5 Extraction of DNA from an agarose gel

DNA fragments separated by agarose gel electrophoresis (see 2.8.3.1) were excised from the gel using a scalpel blade whilst visualising on a UV trans-illuminator (Ultra-Violet products Ltd. Cambridge, Cambridgeshire, UK). The DNA was then purified from the gel using a Qiaquick gel extraction kit (Qiagen), according to the manufacturer's instructions.

2.8 RNA extraction

2.8.1 *Vaccinium*

2.8.1.1 *CTAB RNA extraction*

Following the method described in Jaakola et al., (2001).

All buffers were made in advance, treated with 0.1% DEPC and autoclaved (See 2.1.7) before use.

1.5µl of CTAB extraction buffer (Appendix A -Solutions and Media recipes) was heated to 65^{o}C in a hot block (AccuBlock digital dry bath, Labet International Inc, New Jersey, USA). *Vaccinium* leaf tissue was ground to a fine powder in the presence of liquid nitrogen using a pre-chilled pestle and mortar. 1.5µl of extraction buffer was added to each tissue sample, the sample and buffer were transferred to a 1.5ml microtube and 2% β-mercaptoethanol (v/v) was added to each. The tubes were then incubated at 65^{o}C for 10 minutes with vortex mixing every 3 minutes. Samples were then centrifuged at 10000g for 10 minutes at 4^{o}C (Beckman Coulter Allegera x-22R centrifuge, Beckman Coulter UK Ltd, High Wycombe, UK). The supernatant was transferred to a new microtube and an equal volume of chloroform isoamyl alcohol (24:1) was added. This was then mixed and centrifuged at 13000g for 5 minutes at room temperature. The top aqueous layer was transferred to a microtube leaving the precipitate and chloroform layer undisturbed, this chloroform step was repeated until no precipitate was formed between the aqueous and chloroform layers. The volume of the supernatant was estimated and 1 quarter of this volume was added in 10M lithium chloride solution was added to each sample, then incubated overnight at 4^{o}C.

Samples were spun down at 18000g for 20 minutes at 4^{o}C and the supernatant removed and discarded. The pellets were then washed with 500µl ice cold 70% (v/v) ethanol then centrifuged at 13000g for 5 minutes. This step was then repeated until no more of the colour from the pellet could be removed each time, with the supernatant being discarded. 100µl of SSTE buffer (Appendix A -Solutions and Media recipes) was added to each pellet and incubated at 65^{o}C until the pellet had dissolved, (there may be some insoluble precipitate left). The RNA was then extracted twice with 100µl of phenol-chloroform isoamyl alcohol in the same way as the chloroform extractions. Two volumes of 100% ethanol were added to each sample and incubated at -80^{o}C for 30 minutes. The samples were then centrifuged at 18000g for 20 minutes at 4^{o}C. The supernatant was discarded and the pellets dried using a vacuum concentrator (5031 Eppendorf UK Ltd. Stevenage, UK) for 1 minute then resuspended in RNAse free water.

2.8.2 *Arabidopsis*

2.8.2.1 ***Qiagen RNeasy kit***

Total RNA was extracted from *Arabidopsis* using an RNeasy kit (Qiagen). Around 30 seedlings were used per preparation when the seedlings reached between 7 and 10 days old. Kit was used according to the manufacturer's instructions with a Qiashredder step after grinding at the beginning and an on the column DNase digestion treatment using RNase free DNase (Qiagen). The RNA was eluted in RNase free water and stored at -80^{o}C.

2.8.3 **Nucleic acid size separation**

2.8.3.1 **Agarose gel electrophoresis**

DNA was separated by size using agarose gel electrophoresis. Gels were made using 1% (w/v) electrophoresis grade agarose (Sigma) in 0.5x TBE buffer (Appendix A -Solutions and Media recipes) which was then melted in a microwave oven. The gel solution was then left to cool before adding ethidium bromide (10mg/ml) to a final concentration of 5µg/ml and poured into a gel tank to set.

0.5x TBE was added to the gel tank as running buffer and DNA samples containing loading buffer (Bioline, 5x DNA loading buffer, Blue) were loaded into the wells along with Hyper ladder 1 molecular weight marker (Bioline) in the end well. The gel was run at 35mA under a constant current for around 1 hour (or until the fragments had separated enough). The gel was imaged using a UV trans-illuminator (Uvitech Limited, Avebury house, Cambridge, UK) at a wavelength of 254nm.

2.8.4 **Nucleic acid quantification**

2.8.4.1 ***Nanodrop***

Undiluted RNA or DNA concentrations were measured using a ND-1000 UV-Vis Spectrophotometer (Nanodrop technologies, Wilmington, Delaware, USA) using either water or elution buffer as a blank according to the manufacturer's instructions.

2.9 cDNA synthesis

2.9.1 *Vaccinium*

2.9.1.1 cDNA synthesis for real time PCR

Using a QuantiTect reverse transcription kit (Qiagen), according to manufacturer's instructions. 1ug of RNA from *Vaccinium* was made up to 12µl in RNase free water and 2µl of gDNA wipe-out buffer 7x was added. Controls of no RNA, no enzyme and no DNase treatment were all set up in parallel. The samples were then incubated at 42^{o}C for 2 minutes then at 95^{o}C for 3 minutes before being put on ice. 4µl of Quantiscript reverse transcript RT buffer 5x was added to each sample with 1µl of primer mix and 1µl of Quantiscript reverse transcriptase, (where no RNA or enzyme was used an equal volume of RNase free water was used). The samples were incubated at 42^{o}C for 30 minutes then at 95^{o}C for 3 minutes to denature the enzyme. Samples were stored at -20^{o}C until needed.

2.9.1.2 Full length cDNA synthesis

Using the RevertAid premium first strand cDNA synthesis kit (Thermo scientific) according to the manufacturer's instructions. 1µg of RNA from each *Vaccinium* extraction was used made up to a total volume of 13µl with nuclease free water. 1µl of oligo DT and 1µl of 10mM dNTP mix was added to each sample and incubated at 65^{o}C for 5 minutes before being put on ice. 4µl of 5xRT buffer and 1µl of RevertAid Premium Enzyme Mix was added to each reaction before mixing and incubating at 50^{o}C for 30 minutes and then 85^{o}C for 5 minutes to end the reaction. All of the cDNAs were stored at -20^{o}C.

2.9.2 *Arabidopsis*

2.9.2.1 cDNA synthesis for real time PCR

Using a High Capacity Reverse transcription kit (Applied Biosystems) and following the manufacturer's instructions. 1µg of *Arabidopsis* RNA was used and made up to a total volume of 10µl with nuclease free water. A master mix was made containing (per reaction) 2µl of 10xRT Buffer, 0.8µl of 25xdNTP mix (100mM), 2µl of 10x RT random primers, 1µl of multiscribe reverse transcriptase and 4.2µl of nuclease free water. 10µl of master mix was added to each RNA dilution to give a total volume of 20µl, (no enzyme and no RNA controls were also set up substituting in an equal volume of nuclease free water). All samples were run on a programme of 25^{o}C for 10 minutes, 37^{o}C for 120 minutes and 85^{o}C for 5 minutes before being stored at -20^{o}C until needed.

2.10 Amplification of DNA fragments by Polymerase chain reaction (PCR)

2.10.1 DNA polymerases

For general PCR reactions either BioTaq or BioTaq red (Bioline) were used. For high fidelity amplifications a proof reading Taq Phusion DNA polymerase (Finnzymes, Keilaranta, Finland) was used.

2.10.2 Oligonucleotide primers and reaction mixes

Primers were designed by eye to be around 20bp in length and between 40 and 60% guanine (G) and cytosine (C). All primers were ordered from Invitrogen (Appendix C –List of primers). Reaction mixes were made up following the manufacturer's instructions using buffers and $MgCl_2$ provided.

2.10.2.1 PCR conditions

Table 2.2: Conditions used to run PCR programmes .

PCR conditions					
Stage	Basic	GradVvi	GradVvi (Esk)	GradVvi (Hos1)	Phusion
Initial Denaturation	94°C 5 minutes	95°C 10 minutes	94°C 5 minutes	94°C 5 minutes	98°C 30 seconds
Denaturation	94°C 1 minute	95°C 30 seconds	94°C 1 minute	94°C 1 minute	98°C 10 seconds
Annealing	55°C 46 seconds	55°C 30 seconds	59°C 46 seconds	58°C 46 seconds	55°C 46 seconds
Extension	72°C 1.5 minutes	72°C 1 minute	72°C 1.5 minutes	72°C 1.5 minutes	72°C 30 seconds
Final Extension	72°C 10 minutes	72°C 10 minutes	72°C 10 minutes	72°C 10 minutes	72°C 10 minutes
Number of Cycles	30	30	30	35	20

2.10.3 **5'3' RACE**

Using a 5'/ 3' RACE kit, 2nd Generation (Roche diagnostics Ltd, West Sussex, UK) according to manufacturer's instructions.

2.10.4 **5' RACE**

First strand cDNA synthesis: cDNA, synthesis buffer, deoxynucleotide mixture, specific primer 1 (Appendix C –List of primers), RNase free water and Transcriptor Reverse Transcriptase were added to 1µg of *Vaccinium* total RNA and incubated for an hour at 55°C and then for 5 minutes at 85°C.

The cDNA was then purified using a High Pure PCR Product Purification Kit (Roche diagnostics Ltd.) following the manufacturer's instructions. Binding buffer was added to the cDNA and mixed before adding it to a High pure filter column, spun down to allow the cDNA to bind to the column membrane. The membrane was washed by adding appropriate amounts of wash buffer, spinning it down and discarding the flow through, the membrane and column were then dried and the cDNA was eluted in 50µl of elution buffer. Poly A Tailing of the first strand cDNA was then carried out on ice straight away to avoid any degradation of the cDNA. To add the Poly A tail to the cDNA 10x reaction buffer and 2 mM dATP were added to the purified cDNA and heated to 94°C for 3 minutes before returning to ice and adding the terminal transferase enzyme and incubating at 37°C for 30 minutes then denaturing the enzyme by incubating at 70°C for 10 minutes.

The area of interest was then amplified by PCR using an Oligo dT-anchor primer and specific primer 2 (Appendix C –List of primers) using a high fidelity Taq polymerase, Phusion Taq (Thermo Fisher) and the specific PCR programme detailed below in Table 2.3: RACE PCR conditions used.

Table 2.3: RACE PCR conditions used.

Stage	Temperature (°C)	Time	Number of cycles
Initial denaturation	94	2 minutes	1
Denaturation Annealing Elongation	94 55 72	15 seconds 30 seconds 40 seconds	10
Denaturation Annealing Elongation	94 55 72	15 Seconds 30 Seconds 40 Seconds[a]	25
Final Elongation	72	7 minutes	1

[a] For each successive cycle the annealing time is extended by 20 seconds

After the first round of PCR amplification 5µl was run on a gel to see if clear bands were visible, often a second round of PCR was needed to amplify enough product to be visible on a gel. The second PCR was set up almost exactly the same as the first but using 1µl of undiluted PCR product with nested primers, (Appendix C –List of primers) a PCR anchor primer provided in the kit and the same PCR programme. After the second PCR cycle the whole 50µl were run on a gel and the clearest bands were cut out, extracted from the gel (see 2.7.5) and sent for sequencing (See 2.11.1.2.).

2.10.5 **3' RACE**

Again using the Roche kit following the manufacturer's instructions. To synthesise the first strand cDNA, synthesis buffer, deoxynucleotide mixture, oligo dT anchor primer, water and transcriptor reverse transcriptase were added to total *Vaccinium* RNA. This was then incubated at 55°C for 60 minutes and then at 85°C for 5 minutes to denature the enzyme. The cDNA was then amplified by adding 1µl of cDNA to a PCR anchor primer, (Appendix C – List of primers), deoxynucleotide mixture, Phusion Taq (Thermo fisher), Phusion HF buffer and water which was then run on the RACE PCR programme. Unlike the 5' RACE nearly all amplifications only needed to be run once to produce enough product to be cut out of the gel and sequenced (See 2.7.5).

2.11 **DNA sequencing**

2.11.1.1 ***ExoSAP DNA purification of PCR products***

The ExoSAP method was used to purify PCR products for sequencing by removing remaining primers and TAQ polymerase by adding Shrimp Alkaline Phosphatase and 10x reaction buffer for SAP (both Fermentas, Thermo Scientific). 5µl of PCR product (from a 20µl reaction) was checked by agarose gel electrophoresis (see 2.8.3.1), this was also used to estimate the amount of DNA present by comparing the brightness of the band to the molecular weight marker used. If only one band was present 0.025µl of exonuclease 1, 0.250µl of Shrimp Alkaline Phosphatase (SAP) and 9.725µl of nuclease free water was added to the remaining 15µl of PCR product. The reactions were then incubated at 37^{o}C for 30 minutes then 95^{o}C for 3 minutes to end the reaction. The samples were then sent for sequencing.

2.11.1.2 ***DNA sequencing reaction***

All sequencing reactions were carried out by the DNA sequencing laboratory, School of Biological and Biomedical Sciences, Durham University.

2.11.1.3 ***Sequence analysis***

Sequences were aligned using Clustal Omega and Jalview (Waterhouse et al., 2009). BLAST 2 (Tatusova & Madden, 1999) was then used to compare sequences as well as the BLAST function on TAIR (www.Arabidopsis.org) and the *Vaccinium* Database (www.*Vaccinium*.org).

2.11.1.4 ***GenBank sequence submission***

The sequences for the three *Vaccinium CBF/DREB1* sequences were submitted to GenBank using the Bank-it tool with the names VmDREB1, VuDREB1 and VviDREB1 the accession numbers are JN254610, JN866911 and JN866912 (Appendix D- GenBank submissions).

2.12 **Measurement of gene expression**

2.12.1 **Real time PCR**

2.12.1.1 ***SYBER green method***

The relative transcript levels of genes of interest were quantified by real time PCR with an Applied Biosystems 7300 real time PCR machine. cDNA made with the AB kit (see2.9.2.1) was diluted 1 in 50 with nuclease free water (Promega) and cDNA made with the Qiagen kit was diluted 1 in 10 with nuclease free water. 5µl of the cDNA dilution was added to 10µl of Syber master mix comprising of (per reaction) 7.5µl of Go Taq qPCR master mix 2x, 0.9µl of each of the forward and reverse primers and 0.7µl of nuclease free water. The diluted cDNA and master mix were added to wells in a 96 well plate (STARLAB UK, Blakelands, Milton Keynes, UK). For each sample to be tested 3 replicate wells were set up. All QPCR primers listed were designed by eye (Appendix C –List of primers). All plates contained a reference gene of PEX4 for *Arabidopsis* and Tubulin for *Vaccinium.*

(GoTaq qPCR master mix contains ROX reference dye at a concentration of 20µl per 1.5ml of master mix).

Gene expression levels were analysed using the $\Delta\Delta C_t$ method (Applied Biosystems). The algorithm used is from Relative Quantitation (RQ) algorithms, Applied Biosystems Real-Time PCR Systems Software, July 2007 as described in the Applied Biosystems user bulletin 2007 (Moffat et al., 2012).

2.12.1.2 ***Taq man method***

The relative expression of CBF2 (from *Arabidopsis*) was measured using Taq man rather than SYBER as the Taq man probes were more efficient at distinguishing between the 4 CBFs in *Arabidopsis* than SYBER primers. Using the same method as for SYBER described in 2.12.1.1 but with the following changes.

6µl of cDNA was added per well to 9µl of master mix comprising of (per reaction) 0.75µl of Taq man probe, 7.5µl of Taq man master mix (Primer Design, Southampton, UK) and 0.75µl of nuclease free water. Taq man probes were bought from AB life technologies, (Thermo-Fisher Scientific Ltd).

2.13 **Cloning of DNA fragments**

2.13.1 Plasmids

Coding sequences were amplified using Phusion Taq (Thermo) see 2.10.1) and cloned using the Gateway entry system Pentr/D-TOPO (Invitrogen). Details and maps of entry vectors and destination vectors are in Appendix B -Ghent vector maps

2.13.2 **Gateway recombination**

Using half volume reactions of Pentr-D-TOPO directional cloning kit (Invitrogen following manufacturer's instructions) and sliver cells (Bioline, DH5α *E. coli* strain) DNA coding sequences were cloned into the Gateway entry vector Pentr-D-TOPO. DNA fragments were transferred from the Gateway entry vector to a Gateway destination vector using LR Clonase^{TM}II Enzyme Mix (Invitrogen). Again following the manufacturer's recommended protocol except for, using half volume reactions. (Appendix B -Ghent vector maps). The recombination reactions made in a total volume of 2.5µl were incubated for a minimum of 4 hours at 25^{o}C. 0.5µl of proteinase K (1µg/µl) was added to denature the clonase enzyme and incubated for 10 minutes at 37^{o}C. 1µl of the recombinant plasmid was then used to transform *E. coli*.

2.13.3 Site directed Mutagenesis

Site directed mutagenesis was carried out using two different methods. The first method used to create the Myrtillus and U1 mutated construct from Winistorfer et al., (1991) uses two primers with a 25 base pair complementary overlap with the mutated bases in the middle then a 14bp non-overlapping sequence on the end of each. Using previously made Pentr-D-Topo vectors for each of the *Vaccinium CBF/DREB1s* the new mutagenesis primers were used to amplify the plasmid with the altered base sequence. Using a Phusion Taq PCR reaction (see 2.10.1) and an extension time of 30 seconds per Kb. The reaction mix was then run on an agarose gel (see 2.8.3.1) and the linear plasmid band was cut out and gel extracted (see 2.7.5) . The gel extracted linear plasmid was then transformed back into competent *E. coli* and spread onto kanamycin LB (see 2.3.2). Colonies formed were grown up in overnight cultures and plasmid preparations carried out (see 2.7.4) before being checked by sequencing (see 2.11.1.2). Successfully mutated plasmids were then transformed into Ghent vectors and then *A. Tumefaciens* (see 2.13.2 and 2.14.1.2).

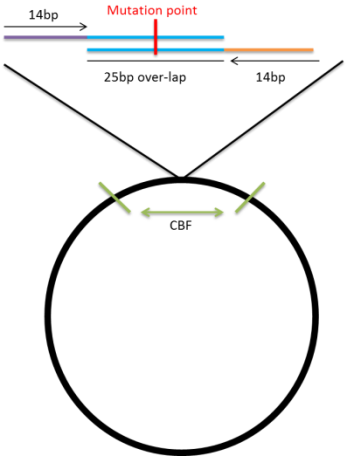

Figure 2-1: Directions of primer annealing and amplification for site-directed mutagenesis by PCR

For three of the constructs, U2, U3 and vitis-idaea a second mutagenesis method was used. Each CBF was amplified from the Pentr-D-Topo plasmid in two separate fragments. Using a primer from the edge of each gene and a mutagenesis primer, and Phusion taq. The two reaction mixes were then run on an agarose gel and the relevant bands gel extracted (see 2.7.5). 1µl of each fragment was added to a tube containing $MgCl_2$ and water as described for a PCR reaction (see 2.10.2) and floated in a beaker of water at 95°C. The water bath was then allowed to cool slowly allowing the fragments slowly to anneal; once the water had cooled to around 40°C, Phusion taq and primers for the outside of the CBF were added to each reaction and the newly annealed fragment amplified by PCR (see 2.10.2). This was then run on an agarose gel, (see2.8.3.1) gel extracted (see 2.7.5) and sequenced (2.11.1.2) before cloning into Pentr-D-Topo (2.13.1) and subsequent transformation into *A. tumefaciens.*

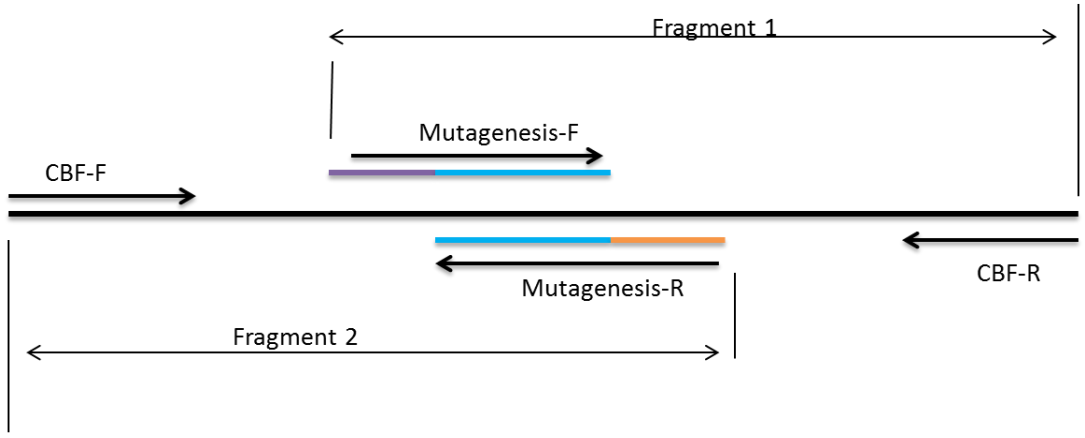

Figure 2-3: Annealing points of primers and direction of amplification for specific primers used in site-directed mutagenesis.

2.13.4 **Restriction digests**

DNA fragments for cloning inserts were produced by restriction digest. The reactions were carried out in the recommended buffer for a minimum of 4 hours at 37°C. Single digests were set up so that the enzyme made up 1/10th of the total reaction volume. Each reaction was carried out in the recommended reaction buffer for the specific enzyme used. Double digests were checked for compatibility and a buffer in which both enzymes would function optimally was found using the New England Biolabs (NEB) double digest finder web page (https://www.neb.com/tools-and-resources/interactive-tools/double-digest-finder).

2.14 **Transformation**

2.14.1 **Bacterial transformation**

2.14.1.1 ***Transformation into competent E. coli cells***

25µl aliquots of DH5α chemically competent *E. coli* (Bioline) were thawed on ice. Between 1ng and 10ng of DNA was then added to each aliquot of cells and left to incubate on ice for 30 minutes. The cells were then heat shocked at 42°C for 30 seconds in a water bath before being returned to ice. 1ml of liquid LB medium was added to each aliquot of cells which were then incubated at 37°C for 1 hour in an incubator with shaking set at around 200rpm. Aliquots of each cell culture were spread onto LB agar plates containing the appropriate selection antibiotics and were incubated overnight at 37°C. Successful transformation plates containing colonies were then sealed and stored at 4°C

2.14.1.2 ***Transformation of A. tumefaciens***

100µl aliquots of frozen *A. tumefaciens* strain C58C1 were defrosted on ice. 1µg of DNA was added to each aliquot and incubated on ice for 30 minutes. The cells were then heat shocked for 5 minutes at 37°C in a water bath before returning to ice. 1ml of liquid LB medium was then added to each aliquot and incubated for 4 hours at 28°C before spinning down the cells in a microfuge. The cell pellet was resuspended in 100µl of LB and spread onto LB agar plates containing the appropriate antibiotics for selection. (See 2.3.3) (Appendix B -Ghent vector maps). Plates were incubated at 29°C for 48 hours before being sealed and stored at 4°C.

2.14.2 Plant Transformation

2.14.2.1 ***Stable transformants***

2.14.2.1.1 ***A. tumefaciens* mediated transformation of *A. thaliana* by floral dip**

Wild type *Arabidopsis* seedlings were grown on MS plates (see 2.3.1) until they were 7 days old. The seedlings were then transferred onto large (42mm) peat plugs with three seedlings per peat plug. When the plants began to flower the flower bolts were clipped to encourage multiple secondary flowering stems to develop. Seven days after clipping the primary bolts, 5ml overnight cultures of *A. tumefaciens* containing the construct of interest were set up with appropriate selection antibiotics (see 2.3.3). 1ml of the overnight culture was then used to seed a 200ml overnight culture again with the appropriate selection antibiotics, the culture was incubated overnight at 29^{o}C with agitation. The *Agrobacterium* were then pelleted down by centrifugation at 3500g for 15minutes at room temperature. The bacterial pellet was then resuspended in 200ml of 5% sucrose solution (w/v) and 0.05% Silwet L-77 (v/v).

The flower stems of each *Arabidopsis* plant were dipped into the *A. tumefaciens* suspension for a few seconds before being placed on their side in a tray lined with paper towel which was then covered with cling film and returned to the growth chambers overnight out of direct light. The next day the plants were returned to their original trays (upright) and watered from below to avoid washing off the bacteria.

2.14.2.2 ***Selection of transformants***

2.14.2.2.1 **BASTA selection**

Seeds with BASTA as a selectable marker were sown on sand and perlite. After seven days the seedlings were sprayed with 150 mM BASTA herbicide (Bayer-Crop Science, Cambridge, UK) at 5 day intervals. Transformants were then transferred to soil.

2.14.2.2.2 **Kanamycin selection**

MS agar plates containing Kanamycin were made as in 2.3.1. Seeds were sterilised by bleach surface sterilisation (see 2.1.8.2) and stratified for 3 days at 4^{o}C. The plates were then air dried in the laminar flow hood, sealed with micropore tape (3M, Bracknell, UK) and put into the light in the percival incubator (Sanyo electric co., ltd) for 6 hours. The plates were then wrapped in tin foil to eliminate light and returned to the percival incubator.

After 48 hours the plates were unwrapped and returned to the light. The transformants were transferred to peat plugs after around 7 days in the light.

2.14.2.3 ***Transient expression***

2.14.2.3.1 ***A. tumefaciens*** **infiltration**

Following the methods described in Clough and Bent, (1998) and Waadt, (2008).

5ml overnight cultures of *A. tumefaciens* containing each of the constructs of interest were set up with appropriate selection antibiotics as described in 2.3.2. 3 week old *Nicotiana benthamiana* plants grown in the conditions described in 2.4.1 were watered generously and covered with a plastic bag to create a humid environment around the plant allowing the stomata to open. This was carried out an hour before starting infiltration. The overnight cultures were spun down at 3000g for 5 minutes in a 15ml tube. Each bacterial pellet was resuspended in 1ml of 10mM $MgCl_2$ solution, and the optical density (OD) of each bacterial suspension was measured using a spectrophotometer. Each bacterial suspension was diluted by adding more $MgCl_2$ to an OD of 0.6. This was then drawn into a 1ml syringe (BD Plastipak™, Oxford, UK) and pushed into the stomata by placing the syringe onto the underside of the leaves of the *N. Benthamiana* plants and placing my thumb on the top of the leaf in the same area as the syringe taking care cause minimal damage to the leaves. The plants were then returned to the growth chambers for 48 hours before imaging (see 2.15.2)

2.14.2.3.2 ***A. tumefaciens*** **co-infiltrations**

To visualise protein interactions *in vivo* co-infiltrations of two constructs were set up. Either one of the *Vaccinium* GFP-VCBF constructs was co-infiltrated with CRT/DRE::LUC (DRE element bound to Luciferase) (Whalley et al., 2011) or a control of cytosolic GFP and CRT/DRE::LUC. The method is the same as in 2.14.2.3.1, although the different *Agrobacterium* constructs were each diluted to an OD of 0.3 and equal amounts of each *Agrobacterium* construct were mixed together in a tube before being drawn into the syringe.

2.15 **Imaging**

2.15.1 **Fluorescence / GFP imaging**

Transient GFP fluorescence in tobacco was imaged by mounting 1cm squares of leaf tissue from around the infiltration site onto glass slides using water as a mounting medium. A

cover slip which was taped to the slide at opposite edges to create a flatter surface for imaging. The fluorescence was then imaged using a Zeiss Meta 510 CLSM (Confocal laser scanning microscope, Carl Zeiss, Cambridge, UK) at a magnification of x40 (oil immersion lens) and the accompanying software.

2.15.2 Luminescence imaging

Luciferase imaging of the *N. benthamiana* co-infiltrations was performed by spraying each infiltrated leaf with 5mM potassium luciferin (Melford laboratories Ltd) and 0.01% Triton X-100 (v/v). The leaves were then put onto the camera stage with a glass plate on top and imaged using a Photek photon-counting camera (Photek, Hastings, UK).

2.15.3 *Arabidopsis* freezing assay images

Each plant for the freezing assay was photographed before freezing and 3 days after freezing using a Canon EOS 550D DSLR set on Raw format and held by a camera stand so that each image has exactly the same zoom and distance from the camera, each photo also contained a 1cm square of white paper in the corner. By setting the camera to Raw format tagged image file format (TIFF) images were produced which allowed the average diameter for each plant to be calculated. Three measurements were taken between the widest possible diameters for each plant. The diameter was measured using AxioVision software (Carl Zeiss, Cambridge, UK) and standardised between photos using the scale bar in the corner of each photo.

2.15.4 Chlorophyll fluorescence measurements

The ratio of variable fluorescence (F_v) over maximal fluorescence (F_m) (F_v/F_m) can be measured as a quantitative indicator of damage to photosynthesis due to freezing stress (Maxwell & Johnson, 2000, Oxborough, 2004). The ratio of F_v/F_m for each plant was measured before and 3 days after being transferred to the freezing chamber as part of the *Arabidopsis* freezing assays. The plants were dark-adapted for 30 minutes before measurements, then the F_v/F_m ratio was measured using a FluorCam 700 mf (Photon Systems instruments, Brno, Czech Republic) on the F_o, F_m and Kautsky effect setting. All measurements used the same parameters for F_o and $_{Fm}$ measurement, Kautsky induction, and dark relaxation before and after Kautsky induction.

2.16 Protein Analysis

2.16.1 Strep-tagged protein pull downs

Two Aracon trays of strep-tagged *Arabidopsis* plants were used for each pull down (42-84 plants). The plants were harvested and flash frozen in liquid nitrogen, then ground to a fine powder in a pestle and mortar whilst still frozen. 3-4 times the volume of the tissue was added in extraction buffer (Appendix A -Solutions and Media recipes), mixed and left on ice for 10 minutes. The solution was then filtered through 2 layers of Mira cloth (Millipore, Merck chemicals Ltd, Nottingham, UK) and centrifuged at 15000g for 20 minutes at 4^{o}C discarding the pellet and re-centrifuging the supernatant. Again the pellet was discarded and the supernatant was then filtered using a millex filter (Millipore) to remove any small debris. Avidin was added to the solution to give a final concentration of 5µg ml^{-1} to remove endogenous biotin or biotinylated proteins. Using a peristaltic pump (set up in the cold room at 4^{o}C) the StrepTactin MacroPrep H-Pr, 1ml column (IBA GmbH, Gottingen, Germany) was washed and equilibrated with cold buffer (Appendix A -Solutions and Media recipes). The sample was then slowly run through the column, the bound protein was eluted from the column by running through a buffer containing 2.5mM desthiobiotin (Appendix A -Solutions and Media recipes) and collecting the flow through in 1ml elutions. The eluted protein was flash frozen and stored at -20^{o}C. The column was regenerated by running TBS containing 1mM HABA (2-(4'-hydroxy-benzeneazo)-benzoic acid) through the column until the colour has changed from yellow to red and back to yellow again.

2.16.2 GFP tagged pull downs

Using a method adapted from Roux et al., (2011).

N. benthamiana leaves were infiltrated with mutant and wt *Vaccinium* GFP-CBF/DREB1 constructs at an OD of 0.6 (as described in 2.14.2.3.1). Half of a leaf per construct was harvested after 48 hours and flash frozen in liquid nitrogen. The tissue was ground to a fine powder using a frozen mortar and pestle and transferred to a pre-chilled 50ml tube. 2ml Extraction buffer (Appendix A -Solutions and Media recipes) per gram of tissue was added to each sample and mixed by inverting. The samples were then centrifuged at 20000rpm for 20minutes at 4^{o}C. The supernatant was then removed and filtered through miracloth (MERK) into a pre-chilled 50ml tube, then filtered through a 0.2µm filter (VWR International Ltd, Leicestershire, UK) into a chilled 15ml tube. The concentration of all of the samples was adjusted to be the same in each sample by quantifying with a Bio-Rad Dc protein assay (Bio-Rad, see 2.16.4). 1.3ml of protein was then added to a 1.5ml microtube

and 130µl of GFPTrap beads (ChromoTek GmbH, München, Germany) and buffer (made as a master mix of 100µl of buffer and 30µl of beads per sample) were added to each sample. The samples were then incubated on a roller for 4 hours at 4°C. To pellet the beads the samples were centrifuged at 500g for 2 minutes and the supernatant removed. 1ml of extraction buffer (without PVPP) was then added to wash the beads and spun down at 500g, the wash step was repeated until the colour had been removed. The last wash was carefully removed with a syringe and needle (BDH laboratory supplies) and 50µl of SDS loading buffer (Appendix A -Solutions and Media recipes) with mercaptoethanol was added. The samples were then heated to 70°C for 20minutes and loaded onto a 12% acrylamide gel run for 1.5 hours and then silver stained (see2.16.6).

2.16.3 Acetone protein precipitation

Using a method from Chivasa et al., (2009).

To precipitate the protein eluted from the strep column, 50µl of eluate was added to 1/10th volume of 1.5M Tris-HCl (pH 8.8), and mixed before adding 4 times the total volume in cold acetone. The solution was mixed and incubated overnight at -20°C.

The samples were then removed from the freezer and centrifuged for 1 hour at 3900g and the supernatant was carefully discarded. The protein pellet was washed twice in 80% acetone (v/v) by semi resuspending the pellet then centrifuging at 13000g for 5 minutes. The washed pellets were then air dried for ~15 minutes and resuspended in SDS loading buffer (Appendix A -Solutions and Media recipes) and stored at 4°C.

2.16.4 Protein quantification assay

Total protein was extracted from *N. Benthamiana* leaf disks as described in Martinez-Garcia et al., (1999) using a Bio-Rad Dc protein assay (Bio-Rad).

2.16.5 Western blots

50ng of protein was boiled in SDS loading buffer and run on 15% polyacrylamide gel for 3 hours. The proteins were then transferred to PVDF membrane (Bio Rad) via semi-dry transfer. The membrane was blocked in 5% milk powder (Marvel, Cadburys, Bourneville, UK) in TBST (Appendix A -Solutions and Media recipes) and incubated with a 1 in 200 dilution of αGFP antibody (#G1112 Santa Cruz Biotechnology Inc. California, USA) in 10% (w/v) milk powder in TBST overnight at 4°C. Anti-rabbit IgG HRP secondary antibody (#E1012 Santa Cruz Biotechnology Inc. California, USA) was incubated for 1 hour at room temperature as a 1 in 5000 dilution in 5% (w/v) milk powder and TBST. The membrane was

then washed and incubated with a West Dura Femto ECL kit (Fisher Scientific, Leicestershire, UK) and bands visualised using a photon counting camera.

2.16.6 Silver staining protein gels

To identify low levels of protein from the GFP downs on an acrylamide gel, silver stain was used. The gel was fixed twice for 30 minutes each time in fixative solution then sensitised by leaving in sensitising solution for 30 minutes. The gel was then washed 3 times in de-ionised water for 10 minutes each wash, and then stained with silver nitrate solution for 20 minutes. The stain solution was washed off with 2x10 minute de-ionised water washes and developed for ~4 minutes in developer solution. The whole reaction was stopped by adding EDTA solution and incubating for 1h. The gel was then washed in milliQ water (MERK) and imaged using a scanner, (Epson (UK), Hertfordshire, UK). Details of all of the buffers and solutions used are listed in Appendix A.

2.16.7 Imperial staining protein gels

To check the protein bands on polyacylamide gels, imperial protein staining was used. The gel was washed for 5 minutes in de-ionised water then submerged in Imperial™ Protein Stain (Thermo Scientific) for 5 minutes before removing the stain. The gel was then washed 3 times in de-ionised water to remove any remaining stain in the tray. Washes in de-ionised water were repeated as necessary until the protein bands became visible.

3 Chapter 3 – Identification and characterisation of *Vaccinium CBF/DREB1*.

3.1 Introduction

Low temperature is a major factor in determining crop cultivation areas (Knight & Knight, 2012). Climate change is making the weather more unpredictable therefore leading to increasing numbers of crops being damaged each year by unexpected freezing events (as described in chapter 1). Cold acclimation is an essential part of enabling plants to survive freezing temperatures (Xin & Browse, 2000) and has been well characterised in *Arabidopsis* (Gilmour et al., 1988). However, relatively few studies have investigated cold acclimation in freezing tolerant species.

Vaccinium species make up a large proportion of forest floor plants in Scandinavia, (Kardell, 1980) also making up large proportions of the dwarf shrubs found in sub-Arctic regions (Press et al., 1998). A better understanding of cold acclimation in freezing tolerant species such as *V. myrtillus*, *V. uliginosum* and *V. vitis-idaea,* whose ability to cold acclimate is essential to their survival, could potentially further highlight the importance of cold acclimation pathways identified in *Arabidopsis*. Alternatively, studies on freezing tolerant species may identify different mechanisms of cold acclimation leading to the improved freezing tolerance found in these species.

C-repeat binding factor (*CBF*) is one of the most well characterised groups of transcription factors associated with cold acclimation in *Arabidopsis,* (Cook et al., 2004, Fowler et al., 2005, Thomashow et al., 2001) as it is activated within 15 minutes of exposure to cold (Gilmour et al., 1998, Liu et al., 1998). *CBF* is also commonly known as dehydration responsive element binding protein 1 (*DREB1*) (Shinwari et al., 1998). *CBF/DREB1* plays an essential role in cold acclimation in *Arabidopsis* through the activation of cold on-regulated genes (COR genes) (Gilmour et al., 1998).

The aims of the work described in this chapter were to establish whether there were differences between the protein sequence of the three *Vaccinium* species, by isolating and sequencing the *CBF/DREB1* genes from each of the three *Vaccinium* species. The next question posed by the work in this chapter was to establish whether the *Vaccinium CBF/DREB1s* were functional in *Arabidopsis*. Functional characterisation of the three *Vaccinium* CBF/DREB1 transcription factors was carried out using transgenic lines of

Arabidopsis overexpressing each *Vaccinium CBF/DREB1,* to establish whether the differences in sequence between the three species resulted in different phenotypes

3.2 Results

3.2.1 Cloning *CBF/DREB1* from Arctic *Vaccinium*

As the genome of any species of *Vaccinium* had not yet been sequenced in 2010 (and is still not available) the first part of the project was to isolate and sequence the *CBF/DREB1* coding sequence from the three Arctic species of *Vaccinium*: *V. myrtillus*, *V. uliginosum* and *V. vitis-idaea*. This was carried out using PCR primers described in Wang et al., (2010) to try to amplify the *CBF/DREB1* sequences from the three Arctic species. The amplified *CBF/DREB1* sequences were cloned, sequenced and submitted to Genbank with the names and accession numbers VmDREB1, VuDREB1 and VviDREB1 JN254610, JN866911 and JN866912 (Appendix D- GenBank submissions).

3.2.2 Sequence comparisons of *CBF/DREB1*

Comparison line-ups were created using Clustal W (Larkin et al., 2007), comparing the *CBF/DREB1* sequences from each of the three Arctic *Vaccinium* species as well as the previously published *V. vitis-idaea* sequence from China (Wang et al., 2010), *V. corymbosum* sequence *(Polashock* et al., *2010),* and the sequences of CBF1-3 from *Arabidopsis* (Gilmour et al., 2004, Stockinger et al., 1997) (see Figure 3-1) .

The Chinese accession of *V. vitis-idaea* has 5 amino acids different to the Arctic *V. vitis-idaea* sequence, *V. corymbosum* has between 9 and 12 amino acids different to the Arctic species of *Vaccinium* and is closest in sequence to *V. uliginosum*. Both *V. vitis-idaea* sequences potentially have an extra 19 amino acids at the start of the sequence relative to the other *Vaccinium* and *Arabidopsis* sequences. There is a relatively low level of conservation in sequences between *Arabidopsis* and *Vaccinium* until the beginning of the cold on regulated (*COR*) gene binding domain after which there is a relatively high level of sequence conservation. It was possible to identify the *COR* gene binding domain in the *Vaccinium* sequences, and the AP2/EREBP signature sequence and DSAWR recognition sequences (Canella et al., 2010) were identified within this region in the *Vaccinium CBF/DREB1* sequences using the annotated *Arabidopsis* sequence due to the high level of conservation.

All three *Arabidopsis CBF/DREB1* sequences have an extra 10 amino acids between the *COR* gene binding domain and COOH region which are not present in any of the *Vaccinium* species. *Arabidopsis* has 6 identified hydrophobic domains in the COOH region (Wang et al., 2005); two of these regions are present in all of the *Vaccinium* species.

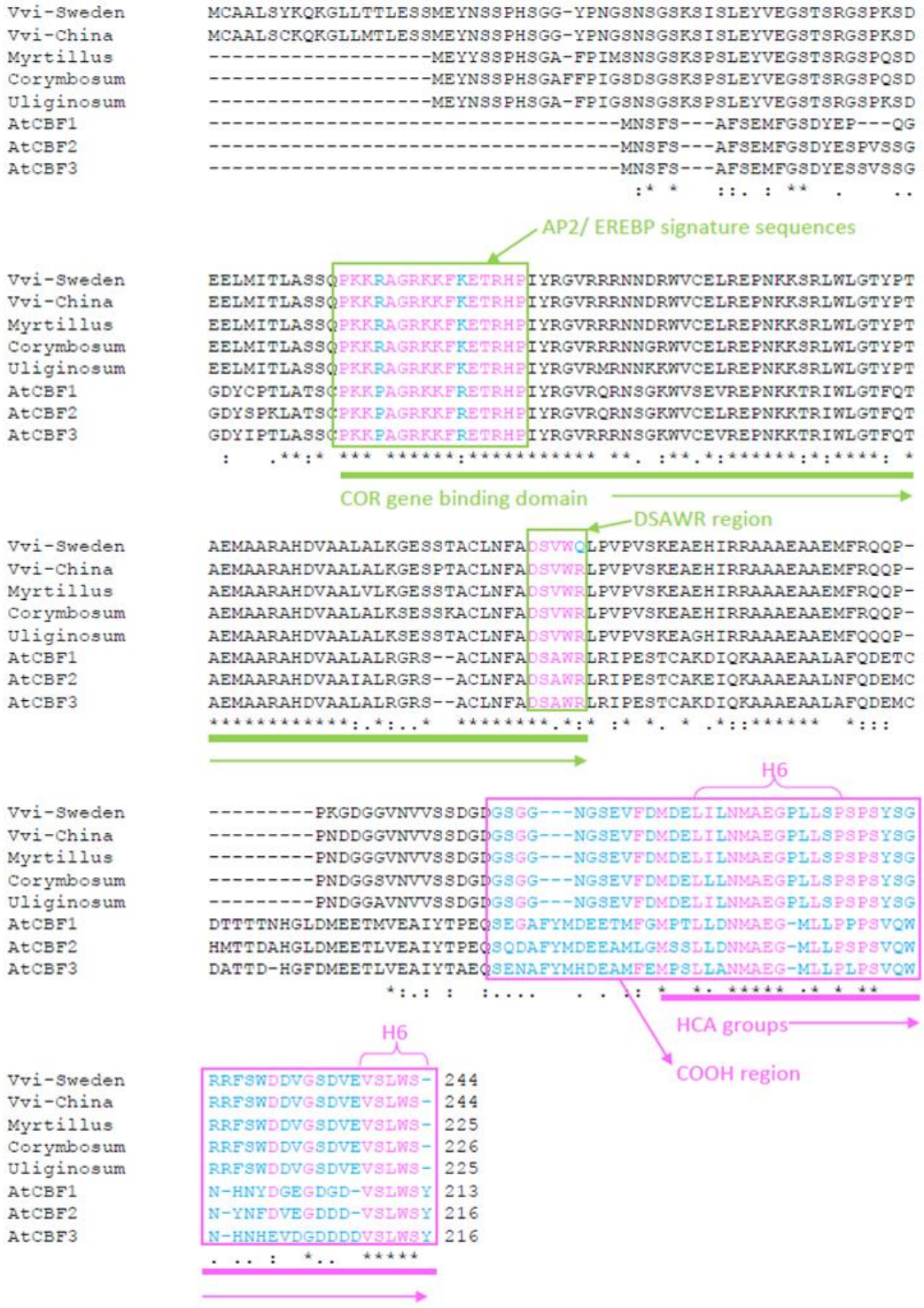

Figure 3-1: Annotated clustal lineups of *CBF/DREB1* from the three Arctic *Vaccinium* species *V. myrtillus* (myrtillus), *V. uliginosum* (uliginosum) and *V. vitis-idaea* (Vvi-Sweden) with the sequences from Chinese *V. vitis-idaea* (Vvi-China), *V. corymbosum* (corymbosum) and CBF1-3 from *Arabidopsis*. *COR* gene binding domains, COOH region and hydrophobic domains (H6) are annotated along with the AP2/EREBP and DSAWR regions.

3.2.3 Over expression of *Vaccinium CBF/DREB1* in *Arabidopsis*

To investigate and compare the potency of the *CBF/DREB1* coding regions, the *CBF/DREB1* from each species was cloned into a 35S expression vector and transformed into Columbia wt. *Arabidopsis*. This allowed the potency of the *CBF/DREB1* coding sequences to be compared in the same genetic background.

3.2.4 Characterisation of the 35S *Vaccinium* CBF/DREB1 transgenic *Arabidopsis*

Seed collected from plants transformed by floral dip were grown on BASTA selection trays (2.14.2.2.1). From the first lines tested it appeared as though the transformation was not successful as no overexpression lines were identified. However, this was due to the BASTA selection method as the *CBF/DREB1* overexpressing plants took around two weeks to germinate which was much slower than the wt *Arabidopsis* seedlings also present in the selection trays. Due to problems with fungus gnat larvae when using the standard BASTA selection method, an altered grown medium of sand and perlite was used for BASTA selection described in 2.14.2.2.1.

A minimum of 15 independent transgenic lines from each construct were tested for *Vaccinium CBF/DREB1* expression. From Figure 3-2A transgenic *Arabidopsis* from all three species can express *Vaccinium CBF/DREB1* to a relatively high level, however, from all of the *V. myrtillus* lines tested there were no lines expressing *CBF/DREB1* to the same high levels of expression that were found in the *V. uliginosum* or *V. vitis-idaea* lines. For this reason high, medium and low expressing lines from each species were selected to test for *COR* gene expression. (NB. There is no expression for the wt control in this experiment as the wt plants were a control to show that the real time primers used for *Vaccinium CBF/DREB1* only show expression of the *Vaccinium CBF/DREB1* and not *Arabidopsis* CBF/DREB1.)

The first *COR* gene tested was *KIN2* (Figure 3-2B) which showed that the *V. myrtillus* CBF/DREB1 lines could induce relatively high *KIN2* expression; however the *V. uliginosum* and *V. vitis-idaea* lines could not induce *KIN2* expression to a level much higher than the wt control plants.

qPCR was then used to test other *COR* genes such as *GOLS3* (Figure 3-2C) and *LTI78* (Figure 3-2D) to determine whether it was just *KIN2* that was expressed to relatively high levels in the transgenic lines expressing the *V. myrtillus* CBF/DREB1 or all *COR* genes. The result was that the *V. myrtillus CBF/DREB1* lines could induce increased *GOLS3* and *LTI78* expression with exactly the same pattern of expression as *KIN2*. The *V. uliginosum* and *V. vitis-idaea*

lines appeared unable to induce an increased in expression of any of the *COR* genes beyond the basal level of expression found in the wt control *Arabidopsis*.

Expression of *COR414* (Figure 3-3A) again showed the same pattern of expression for the *V. myrtillus CBF/DREB1* lines but to a much lower relative level than the other *COR* genes tested. *COR15A* (Figure 3-3B) also showed that *V. myrtillus CBD/DBRE1* overexpression can cause an increase in expression to a high level but additionally, *V. uliginosum* and *V. vitis-idaea CBF/DREB1* could also cause a slight increase in expression to a higher level than the *Arabidopsis* wt.

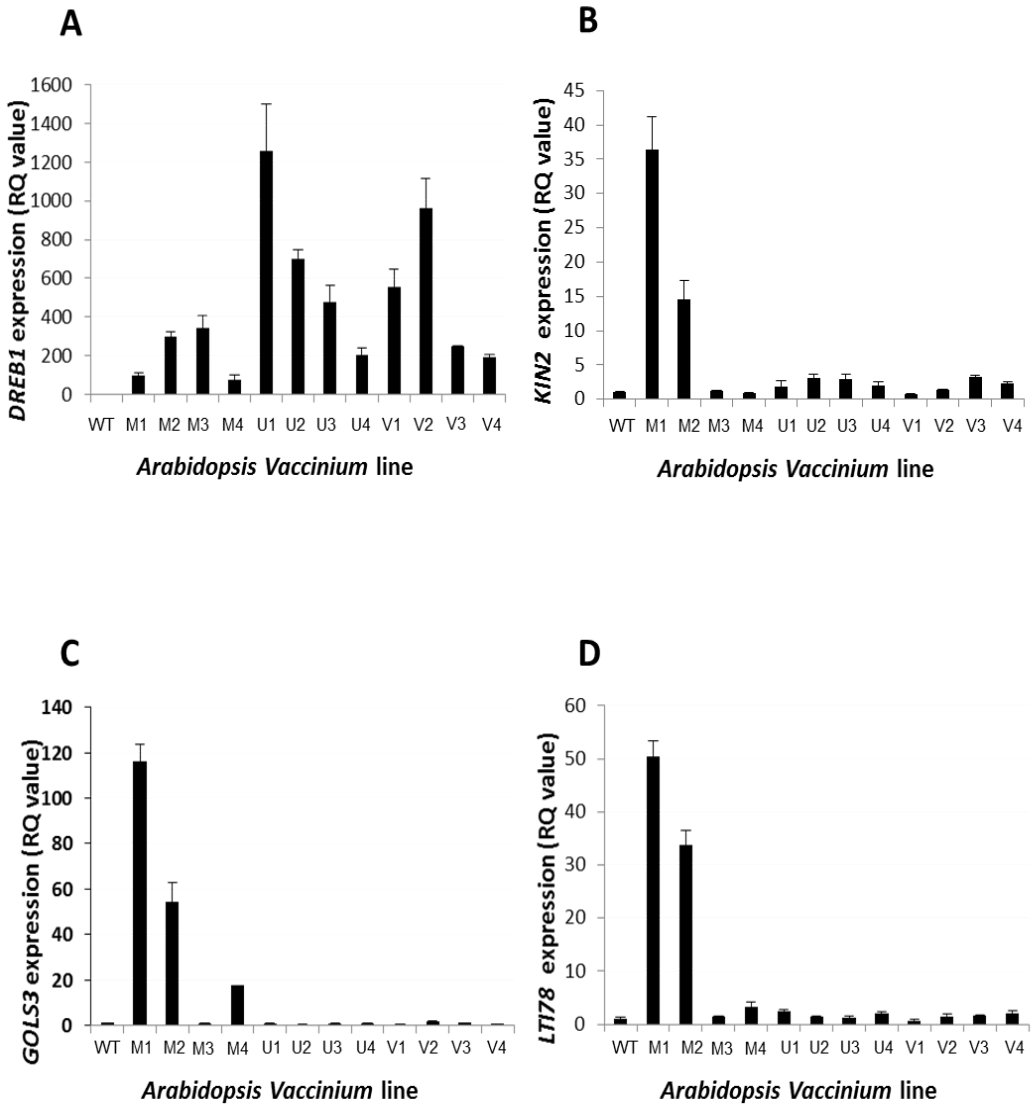

Figure 3-2: Real time expression analysis of transgenic *Arabidopsis* lines expressing 35S- *VCBF/DREB1* coding sequences from *V. myrtillus* (M), *V. uliginosum* (U) and *V. vitis-idaea* (V) for *Vaccinium DREB1* expression(A), *KIN2* expression (B), *GOLS3* expression (C) and *LTI78* expression (D). Samples were normalised to the wt control, error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Student's *t*-test**.**

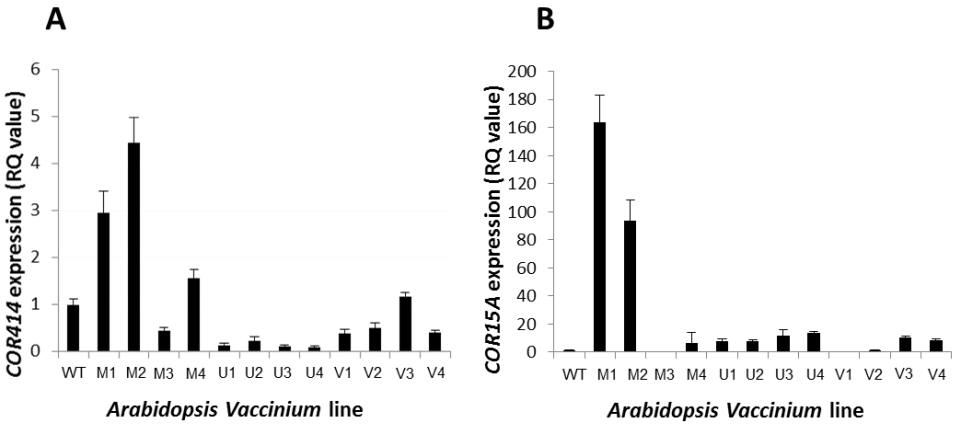

Figure 3-3: Real time expression analysis of transgenic *Arabidopsis* lines expressing 35S-*VCBF/DREB1* coding regions from *V. myrtillus* (M), *V. uliginosum* (U) and *V. vitis-idaea* (V) for *COR414* expression (A) and *COR15a* expression (B). Samples were normalised to the wt control, error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Student's *t*-test.

3.2.5 Phenotypic analysis of *CBF/DREB1* overexpressing transgenic *Arabidopsis.*

The most obvious differences in phenotype between the transgenic plants and the wt was the difference in germination rate as some of the transgenic lines took two weeks to germinate. The *V. myrtillus* lines were also on the whole, smaller than lines expressing *CBF/DREB1* from the other two species and had flatter darker leaves. As well as growing the most slowly, the *V. myrtillus* lines never reached the same size as the *V. uliginosum* and *V. vitis-idaea* construct plants. The *V. myrtillus* construct plants had a delayed flowering phenotype and took four months to fully set seed. The lines expressing the other two constructs didn't appear to grow differently to wt plants.

The size of the plants to be used for the freezing assays were measured, the diameter of each rosette was measured from the three widest possible points and then averaged for each plant. Eight plants for each line and two independent transgenic lines per species were used. (Figure 3-4)

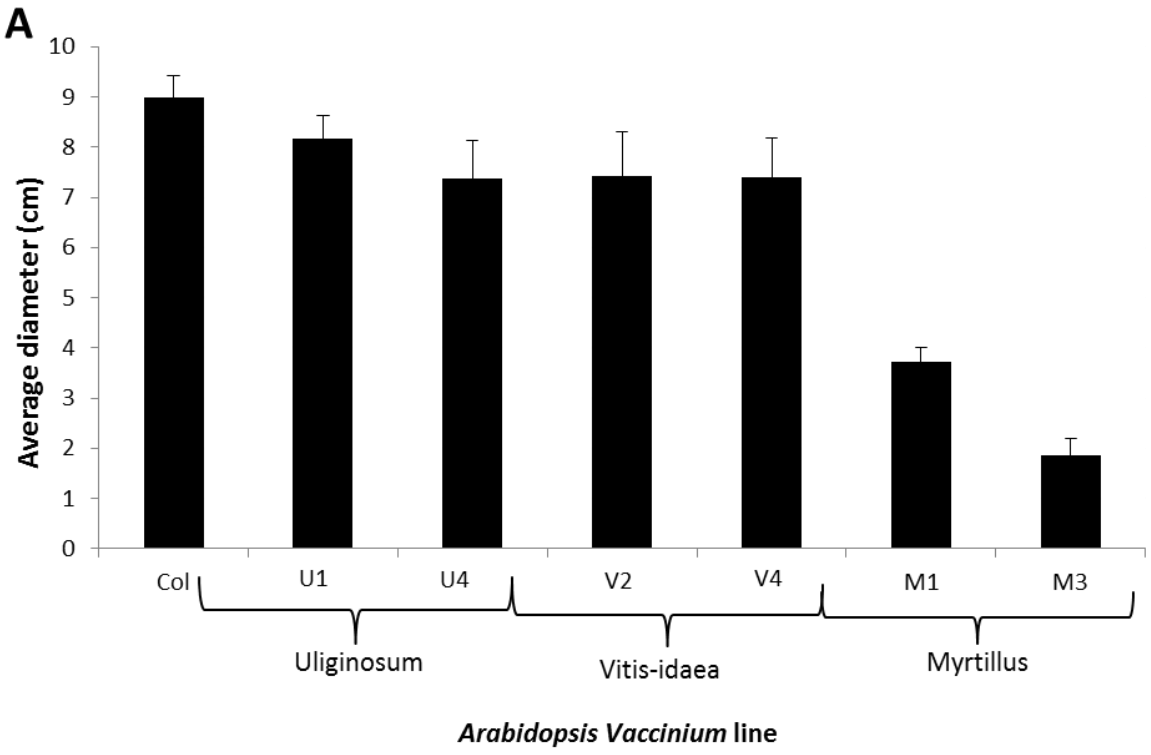

Figure 3-4: Comparisons of average rosette diameter for two overexpression lines for each *Vaccinium CBF/DREB1* and Columbia wt plants (Col), error bars show standard error of the mean.

The average rosette diameter for both *V. myrtillus CBF/DREB1* lines was significantly smaller than the average rosette diameter for the wild type and *V. uliginosum* and *V. vitis-idaea CBF/DREB1* lines. The *V. uliginosum* and *V. vitis-idaea* lines were close in average diameter to the wt *Arabidopsis* plants so were not displaying the same phenotype.

3.2.6 Testing freezing tolerance in the *Vaccinium CBF/DREB1* overexpression *Arabidopsis* lines.

Since the *V. myrtillus* 35S-*CBF/DREB1* lines could induce high levels of *COR* gene expression, freezing assays were carried out to see whether this had led to altered freezing tolerance in the transgenic *Arabidopsis* lines. High and low expressing lines for each of the three *Vaccinium CBF/DREB1s* were chosen for the freezing assays, eight plants of each line and a wild type control were frozen at -7^{o}C without a period of acclimation for 24h in the dark before being returned to 20^{o}C growth chambers. Photos of each individual plant were taken before and after freezing for comparison (Figure 3-5) and the chlorophyll fluorescence of each plant (F_V/F_M) was also measured before and after (Figure 3-6) .

Figure 3-5: Representative images for each overexpression line used in the freezing assay experiments. The top row were taken before freezing and the bottom row 3 days after freezing. For wt (I, VI), and 35S-*CBF/DREB1* lines from *V. uliginosum* (II, VII), *V. vitis-idaea* (III, VIII) and *V. myrtillus* (IV, IX and V, X).

From Figure 3-5, the phenotypic differences between *V. myrtillus* lines and the lines from the other two *Vaccinium* species are clear; two *V. myrtillus* lines were photographed because of the difference in average size between the two lines. From the photographs it appeared that after freezing, the wt, *V. uliginosum* and *V. vitis-idaea* constructs were dead. This was tested using chlorophyll fluorescence measurements before and after freezing (Figure 3-6). Chlorophyll fluorescence could not only indicate whether the plant is dead or alive but also quantify how much damage has been induced by freezing, which will not always visible by eye. From the chlorophyll fluorescence images (Figure 3-6A) the *V. uliginosum* and *V. vitis-idaea* plants appeared dead as there is no visible fluorescence. This was quantified using F_V/F_M (Figure 3-6B) measurements before and after freezing. All eight plants for each line were measured before and after freezing and the results averaged for each line. The results show that the wt, *V. uliginosum*, and *V. vitis-idaea* transgenic lines were dead as negative F_V/F_M values show that there was no chlorophyll fluorescence and therefore no activity at all. The plants from the *V. myrtillus* line with the smaller phenotype

(Figure 3-5 v and x), have almost normal chlorophyll fluorescence (0.8), however, the plants from the other *V. myrtillus* line used, although looking un-damaged had a small amount of freezing damage as the average F_V/F_M had reduced from 0.8 to 0.65 (Figure 3-5 iv and ix). Both *V. myrtillus* lines were able continue in development to flower normally despite being frozen.

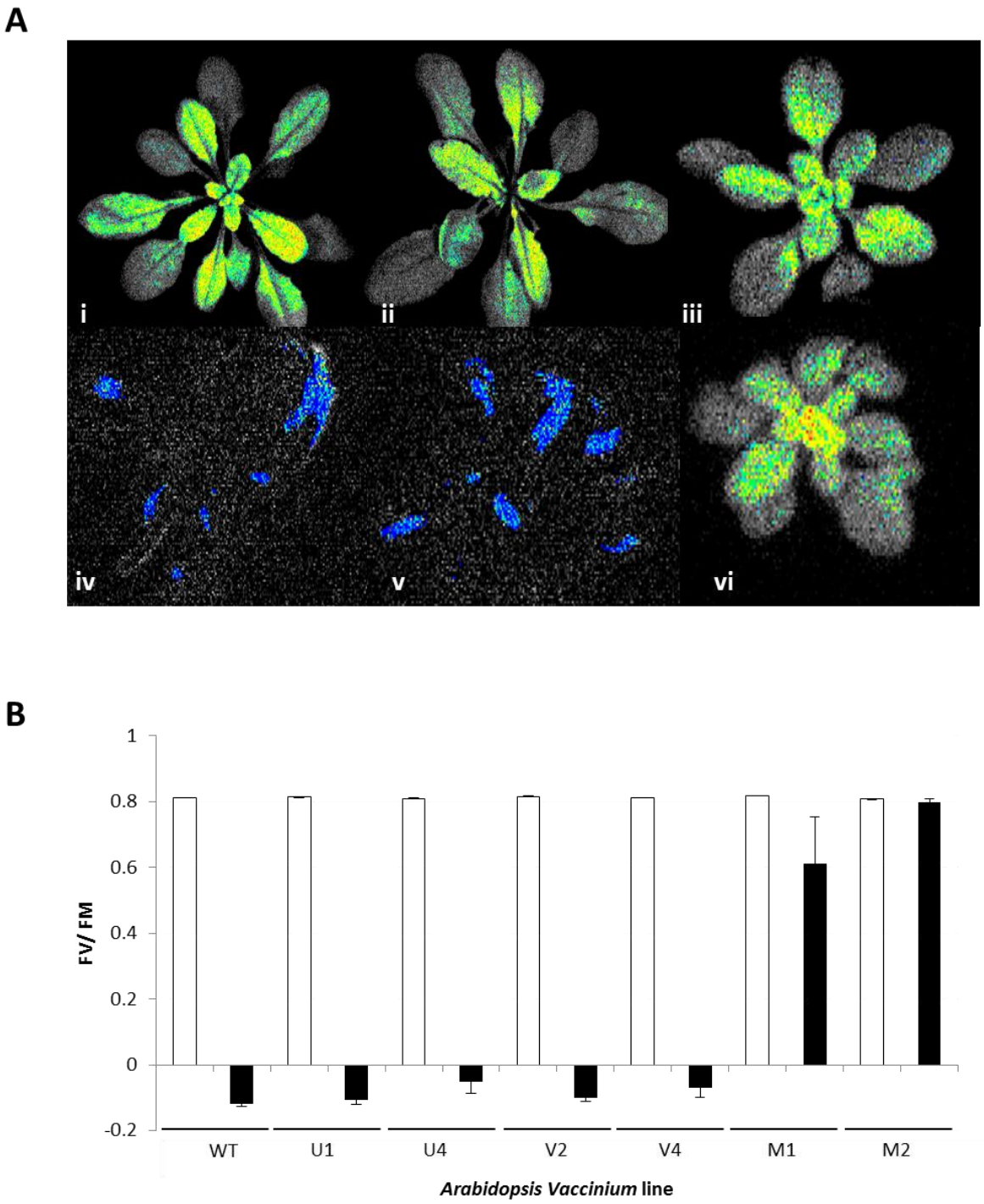

Figure 3-6: Part A, Images of chlorophyll fluorescence taken before (top row) and after (bottom row) freezing at -7^{o}C for *V. uliginosum* (I, iv) *V. vitis-idaea* (ii, v) and *V. myrtillus* (iii, vi) lines. Part B: Measurements of F_V/F_M were taken for each plant before (white) and after freezing (black) and averaged for each transgenic line, wt, *V. uliginosum (U)*, *V. vitis-idaea* (V) and *V. myrtillus* (M). Error bars show standard error of the mean.

3.3 Discussion

3.3.1 Differences in *Vaccinium CBF/DREB1* sequence

One of the most important results is that there was a difference in *CBF/DREB1* sequence between the three species of Arctic *Vaccinium*. The high level of conservation between sequences for all of the *Vaccinium* species and *Arabidopsis* suggests that CBF/DREB1 has an important function or role. Of particular note is the high level of conservation between the species in the *COR* gene binding domain, Canella et al., (2010) suggest that the PKKPAGR sequence at the beginning of this binding domain is essential for *COR* gene binding and any mutation to this sequence impairs the ability of the CBF/DREB1 to bind to *COR* genes. However, all of the *Vaccinium* species have 1 amino acid different to this (PKK**R**AGR) suggesting that small deviations from the sequence still allow *COR* gene binding. It is possible that *Vaccinium CBF/DREB1* would be able to bind *Arabidopsis COR* genes with this amino acid substitution. This could be tested using site directed mutagenesis to substitute the Proline found in the *Arabidopsis* sequence for the Arginine in the *Vaccinium* sequence. However, as the sequences of *Vaccinium COR* genes are not yet known this substitution may prove to be beneficial in *Vaccinium*. This could be tested using site directed mutagenesis of this region of interest. Comparing the induced freezing tolerance of the Chinese *V. vitis-idaea CBF/DREB1* with the Swedish *V. vitis-idaea CBF/DREB1* sequence when overexpressed in *Arabidopsis* could also potentially highlight further amino acid substitutions that may prove beneficial.

Arabidopsis has an extra 10 amino acids between the *COR* gene binding domain and the COOH region that are not present in any of the *Vaccinium* species. It would also be interesting to see if adding this insert to the *Vaccinium CBF/DREB1* would alter freezing tolerance and activity. There are six hydrophobic domains present in the COOH end of the *Arabidopsis* sequence (Wang et al., 2005); of these six hydrophobic domains only two are identifiable in *Vaccinium,* suggesting that adding the extra ten amino acids from *Arabidopsis* would result in an altered shape and would therefore most likely reduce the activity of the protein produced.

3.3.2 Overexpression of *V. myrtillus CBF/DREB1* in *Arabidopsis* induces a dwarf phenotype.

The overexpression of *Vaccinium CBF/DREB1* lines that show a small slow growing phenotype are consistent with the results of Liu et al., (1998) and Gilmour et al., (2004) that show freezing tolerant lines of *Arabidopsis* are small in size due to the increased

amount of energy the plant is putting into becoming freezing tolerant rather than growing large. This requires more energy to carry out processes such as accumulation of compatible solutes that may not be present in the plant under normal conditions. The small phenotype of some of transgenic lines caused a number of problems due to their slow growth rate. The first was (as mentioned above) that the first transformants were actually missed during BASTA selection because of their slow germination time, and a lot of plants were also lost to black fly larvae; this led to a new method of BASTA selection being developed to avoid the same problems with fly larvae again. The overexpression phenotype also meant that the plants produced very few seeds; to make sure that as many seeds as possible germinated, a method of growing seeds on giberellic acid (GA) plates was used until the plants were large enough to transfer to peat plugs. The plants were then dipped in dilute GA weekly until they began to flower. The GA plates method was used to encourage more of the seeds to germinate and in a faster time than the usual two to three weeks. The larger plants were dipped in GA to encourage the plants to flower normally and therefore produce a greater yield of seed in the same time frame. GA has been shown to reverse dwarf phenotypes caused by *CBF/DREB1* overexpression (Hsieh et al., 2002), as overexpression of AP2 transcription factors such as *CBF/DREB1* and *DDF1* (dwarf and delayed flowering 1) have been shown to repress GA biosynthesis (Magome et al., 2004) which is essential for growth and development, seed germination, leaf expansion and flowering time. Hence the dwarf phenotype and delayed flowering time in the *V. myrtillus CBF/DREB1* overexpression lines. Under normal conditions, GA regulates production of DELLA proteins which repress growth; this pathway modulates according to the level of abiotic stress faced and GA is reduced under stress conditions. However, the level of bioactive GA is permanently reduced in the *CBF/DREB1* overexpression lines. As a result a subgroup of genes functioning against abiotic stress are permanently activated (Niu et al., 2014) and therefore more DELLA proteins are produced, causing a dwarf phenotype (Achard et al., 2008).

3.3.3 Differences in freezing tolerance induced by the three *Vaccinium CBF/DREB1s* in *Arabidopsis*.

Interestingly, despite the *V. myrtillus* overexpression lines having the lowest *CBF/DREB1* expression levels of the three species, it has the ability to induce *COR* gene overexpression, whereas *CBF/DREB1* lines from the other two species have much higher *CBF/DREB1* expression levels and no increased *COR* gene expression in *Arabidopsis*. Lines from all three species with lower levels of *CBF/DREB1* expression were also tested for *COR* gene

expression to test whether very high levels of *CBF/DREB1* expression were inhibiting *COR* gene expression in the high expression lines. However, the lower expression lines showed the same result. This was consistent with the results of the freezing assays in that the *V. myrtillus* lines were able to survive freezing without a period of acclimation, whereas the lines from the other two species were too badly damaged by freezing to survive.

It would be interesting to look further at the extent of the differences in freezing tolerance between the three overexpression constructs using a greater range of freezing temperatures, and also to test how much the temperature can be lowered before it has an effect on the survival of the *V. myrtillus* lines. This would establish whether the overexpression of *V. myrtillus CBF/DREB1* had increased the freezing tolerance to the level of acclimated wt *Arabidopsis* or to an improved level of freezing tolerance. It would also have been better to carry out the freezing assays in comparison to an *Arabidopsis CBF/DREB1* overexpression line as a control as well as the wild type lines, so as to establish whether the overexpression of the *V. myrtillus CBF/DREB1* in *Arabidopsis* could induce more freezing tolerance than the overexpression of *Arabidopsis'* own *CBF/DREB1*. This experiment was set up in the original freezing assay experiments, but unfortunately when tested the *Arabidopsis CBF/DREB1* overexpression lines were not overexpressing *Arabidopsis CBF2* as previously thought.

The differences in freezing tolerance between the three overexpression constructs appear to be due to their differing ability to induce *COR* gene expression in *Arabidopsis.* Why there is a difference in the ability of the CBF/DREB1 from each species to induce *COR* gene expression is investigated further in Chapter 4.

4 Chapter 4 – mechanism of *Vaccinium* CBF/DREB1 action

4.1 Introduction

Overexpression of *CBF/DREB1* from *V. myrtillus* caused a dwarf phenotype in the *Arabidopsis* lines produced, which could be rescued by the addition of GA (Chapter 3). This showed that the stress response pathway in the *V. myrtillus* lines was constitutively active (Hsieh et al., 2002, Magome et al., 2004), but not the lines produced from the other two *Vaccinium* species. It has been shown previously that overexpression of any of the three cold induced *CBF/DREB1* genes in *Arabidopsis* causes constitutive freezing tolerance (Gilmour et al., 2004, Jaglo-Ottosen et al., 1998, Sharabi-Schwager et al., 2010) without a period of acclimation. CBF/DREB1 is responsible for activating 12% of *COR* genes (Vogel et al., 2006) which encode proteins involved in a range of different processes that have the combined effect of freezing tolerance (Guy et al., 1985). CBF/DREB1 activates *COR* genes by binding the cold regulatory element (Yamaguchi-Shinozaki & Shinozaki, 1994) which comprises a 9 base pair recognition sequence called the dehydration responsive element (DRE). Within the DRE element there is a 5 base pair sequence known as the C-repeat element (CRT) (Baker et al., 1994). CBF/DREB1 binds to the CRT/DRE element on the promoter region of *COR* genes in response to cold (Stockinger et al., 1997).

In Chapter 3 it was demonstrated that *V. myrtillus CBF/DREB1* can induce increased *COR* gene expression and therefore induce constitutive freezing tolerance in *Arabidopsis,* whereas *V. uliginosum* and *V. vitis-idaea* CBF/DREB1 could not. From the sequences of *CBF/DREB1* isolated from the three *Vaccinium* species it was possible to identify the *COR* gene binding domain present in each *Vaccinium CBF/DREB1* sequence (Canella et al., 2010). There were five differences in amino acid sequence between the three *Vaccinium* species and all three *Vaccinium* CBF/DREB1 sequences were very close in sequence to *Arabidopsis* in the *COR* gene binding domain region (Figure 3-1).

The results presented in Chapter 3 demonstrated that only *V. myrtillus* can induce *COR* gene expression in *Arabidopsis*. The aim of the work described in this chapter was to establish why there was this difference in *COR* gene induction and freezing tolerance between the transgenic lines produced from the three different *Vaccinium* species.

A number of hypotheses were posited to account for this the first of which was; that the differences in *COR* gene induction between the three species are the result of the three CBF/DREB1 proteins produced being targeted to different parts of the cell. Alternatively the observed results could have been due to differential stability of the CBF/DREB1 protein produced by each of the three *Vaccinium* species. A third hypothesis was that small

differences in the protein sequence of the *COR* gene binding domain of the three *Vaccinium* CBF/DREB1 proteins accounts for differential ability to activate *COR* gene expression. This hypothesis was investigated by the characterisation of CRT/DRE binding and activation by CBF/DREB1 proteins produced from each of the three *Vaccinium* species using reporter gene assays. The difference in binding and activation of CRT/DRE was most likely to have been caused by one of five differences in amino acid sequence between the species. These five amino acid positions were then used as targets for site-directed mutagenesis to determine whether each mutation improved the CRT/DRE binding ability of each *Vaccinium* CBF/DREB1. Each point mutation substituted the amino acid to match the sequence of *V. myrtillus* as this had been identified as the most potent CBF/DREB1 of the three.

4.2 Results

4.2.1 Imaging of CBF/DREB1 localisation in *N. benthamiana* using GFP-CBF/DREB1 constructs

The first issue was to investigate the localisation of each expressed CBF/DREB1 protein. This was carried out using constructs expressing each of the three *Vaccinium* species *CBF/DREB1* coding sequences with a 35S promoter and N-terminal GFP tag (method described in 2.13. Each construct was infiltrated into *N. benthamiana* and imaged by confocal microscopy after 48h to establish where the tagged *CBF/DREB1* had been localised to and whether there were differences in the level of GFP.

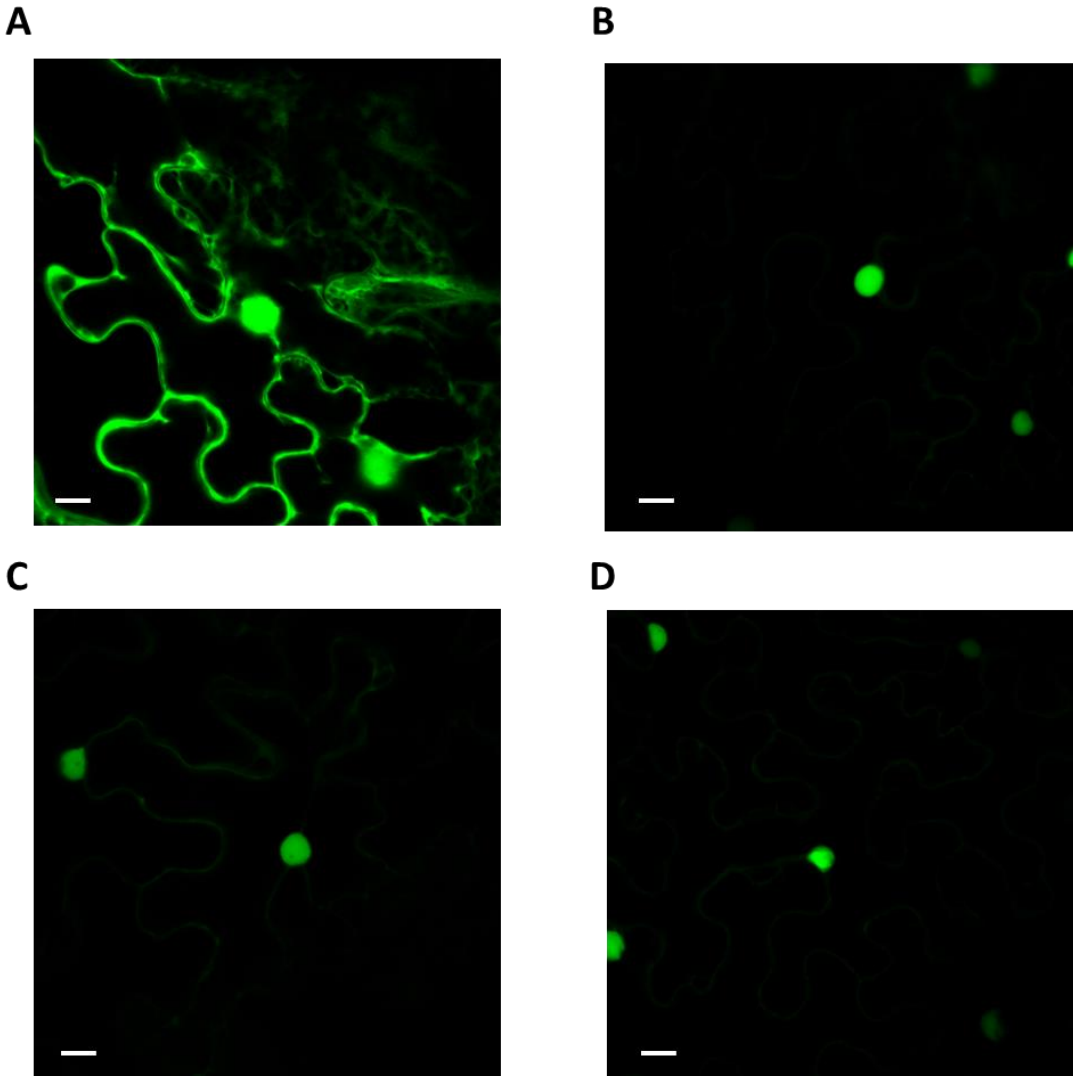

Figure 4-1: Confocal microscopy images (x40) of *Vaccinium* GFP-CBF/DREB1 protein localisation *V. myrtillus* (B), *V. uliginosum* (C) and *V. vitis-idaea* (D) with a cytosolic free GFP control (A). White scale bars represent 22µM.

The confocal microscopy (Figure 4-1) showed all three *Vaccinium* GFP fusion proteins were nuclear localised. The *V. uliginosum* construct was slightly more difficult to image, therefore giving a higher level of background noise when imaging (Figure 4-1C) in

comparison to the other two *Vaccinium* constructs (Figure 4-1 A and D). However, the background fluorescence in Figure 4-1C was significantly less than that in the free GFP control (Figure 4-1A) suggesting it was an artefact due to longer exposure times rather than cytosolic localisation.

4.2.2 Western blot analysis of transient *Vaccinium* GFP-CBF/DREB1 protein expression

The increased difficulty in imaging GFP fluorescence produced by the *V. uliginosum* fusion protein gave rise to the hypothesis that there may be a difference in CBF/DREB1 protein stability between the three *Vaccinium* species, giving different amounts of fusion protein present in comparison.

The three *Vaccinium* GFP-CBF/DREB1 constructs were infiltrated into *N. benthamiana* and leaf disks harvested after 24, 48 and 72h. Total protein was extracted and western blot analysis carried out as described (2.16.5).

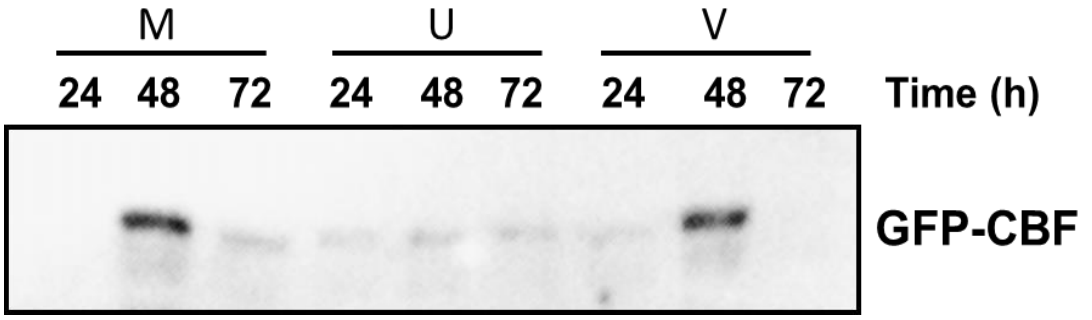

Figure 4-2: Western blot analysis anti GFP antibody to detect *Vaccinium* GFP-CBF/DREB1 fusion proteins extracted from *Nicotiana benthamiana* at 24, 48 and 72h time points after infiltration. Fusion proteins were made for CBF/DREB1 from *V. myrtillus* (M), *V. uliginosum* (U) and *V. vitis-idaea* (V).

There are no available antibodies for anti-CBF/DREB1 so an anti-GFP antibody was used to identify the GFP-CBF/DREB1 fusion proteins at ~50kDa as opposed to 24kDa for free GFP. From Figure 4.2 both the *V. myrtillus* and *V. vitis-idaea* constructs are expressed after 48h; however, relatively little fusion protein was detected for *V. uliginosum*. All of the samples also had bands present for free GFP at 24kDa (not shown).

4.2.3 Reporter gene assays in *Nicotiana benthamiana*

To establish why there is a difference in *COR gene* induction between *V. myrtillus* and *V. vitis-idaea,* reporter gene assays were used. 35S::GFP-VCBF/DREB1 constructs were co-infiltrated into *N. benthamiana* with a CRT/DRE::LUC construct as shown in Figure 4-3. The CRT/DRE::LUC construct (made by Whalley et al. (2011)) is a fusion between four copies of the CRT/DRE binding site for *COR genes* and firefly luciferase. If the GFP-CBF/DREB1 protein

can bind *COR genes* it will be able to bind the CRT/DRE element thus inducing luciferase expression. This expression can be visualised by spraying each leaf with luciferin which causes the luciferase to become luminescent, enabling it to be imaged using a photon counting camera.

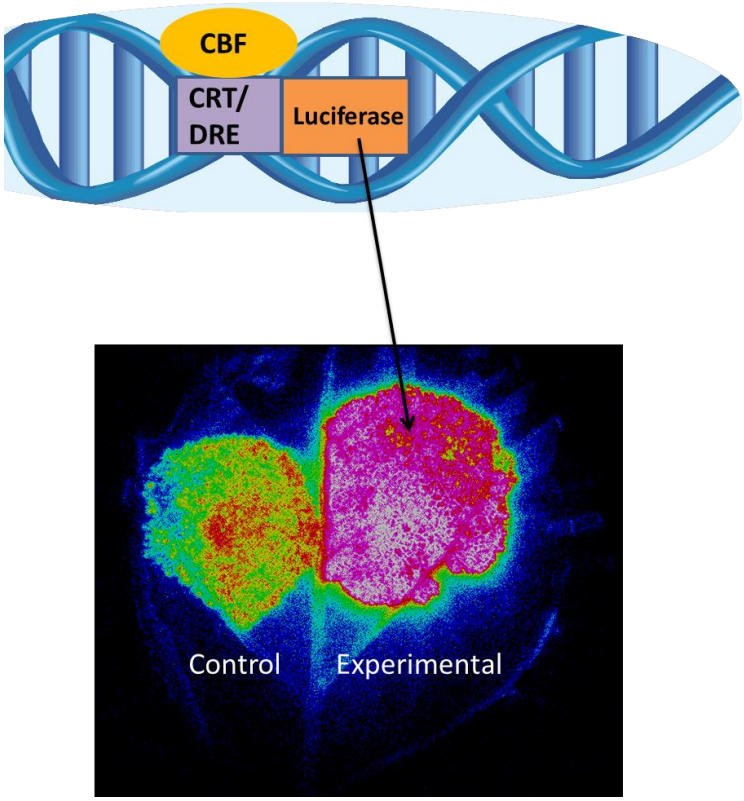

Figure 4-3: GFP-CBF/DREB1 protein binding to the CRT/DRE element when co-expressed caused luciferase expression which was then imaged with a photon counting camera. Cold colours show low expression and warmer colours higher expression. Control constructs of 35S-GFP and the CRT/DRE::LUC construct were co-infiltrated to measure background expression.

Constructs of 35S::GFP-VCBF/DREB1 were produced for all three species and were co infiltrated as a patch on half of each tobacco leaf. A control of 35S-GFP co-infiltrated with CRT/DRE::LUC was used on the other half of each leaf to account for the varying level of expression between leaves. Average luminescence per pixel was measured for each infiltrated leaf patch. To account for the natural differences in expression present between leaves, such as the differences in background chlorophyll fluorescence and background luciferase luminescence for each leaf; the experimental samples were normalised by dividing the value for average luminescence per pixel for the experimental patch, by the value for average luminescence per pixel from the control patch on the same leaf.

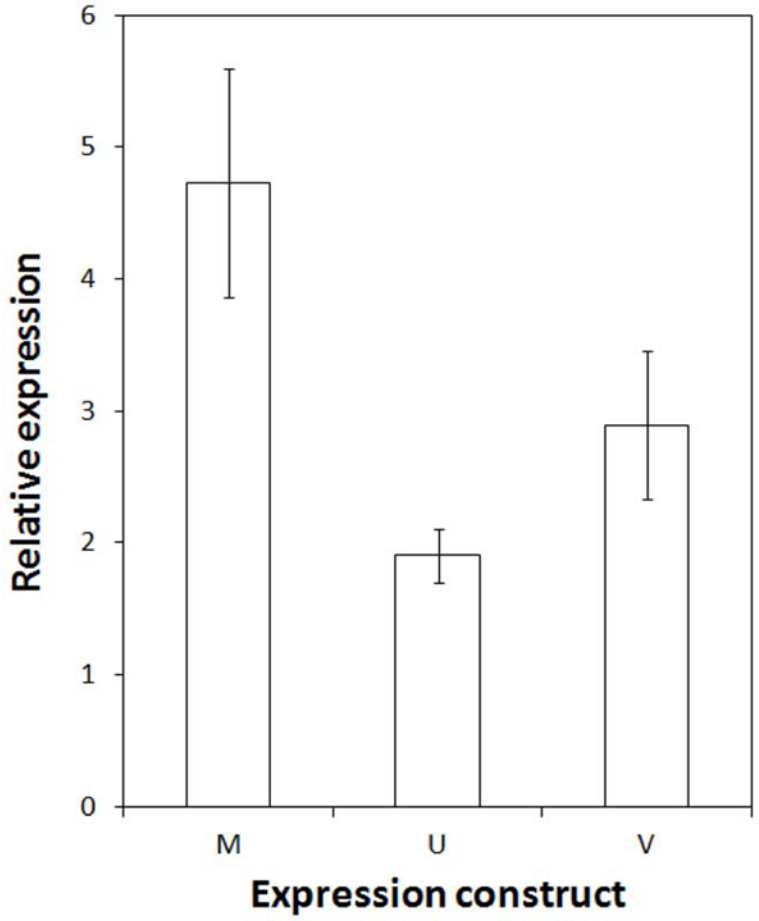

Figure 4-4: Reporter gene assays for *Vaccinium* CBF/DREB1 and the *COR gene* binding site CRT/DRE fused to firefly luciferase coding sequence. Constructs for *V. myrtillus* (M), *V. uliginosum* (U) and *V. vitis-idaea* (V) were compared to a free GFP control. Average luminescence per pixel for each patch was divided by the control background luminescence to give relative expression values. Error bars represent standard error of the mean.

From Figure 4-4 it appears that *V. myrtillus* CBF/DREB1 can activate the CRT/DRE element most efficiently as there is a higher level of expression than with the other two constructs. Both *V. uliginosum* and *V. vitis-idaea* can bind the CRT/DRE element and induce luciferase expression, but not to the same level as *V. myrtillus*.

4.2.4 Site directed mutagenesis of *Vaccinium CBF/DREB1*

As there is a difference in the ability to activate *COR* genes between the three species, it is possible that the five differences in amino acid sequence in the *COR* gene binding domains of the CBF/DREB1 from the three species are responsible. To test this hypothesis site directed mutagenesis reactions were set up (Figure 4-5). As *V. myrtillus* can activate *COR* genes the mutations were to change each amino acid that differed in *V. uliginosum* and *V. vitis-idaea* to become the same amino acid as *V. myrtillus* and test these changes with the same reporter gene assay. As *V. myrtillus* had one amino acid different to the other two *Vaccinium* species, this mutation was made in *V. myrtillus* to assess changes as it only

required one mutagenesis construct to test this substitution rather than two. Specific amino acid substitutions carried out for each of the five constructs produced are listed in Table 4.4.

Table 4.4: Site directed mutagenesis amino acid substitutions for each mutant CBF/DREB1 made.

Species	**Mutation**
V. myrtillus	Val114 to Ala **114**
V. uliginosum 1	Met93 to Arg93
V. uliginosum 2	Lys97, Lys98 to Asp97, Arg98
V. uliginosum 3	Ser117 to Gly117
V. vitis-idaea	Gln151 to Arg151

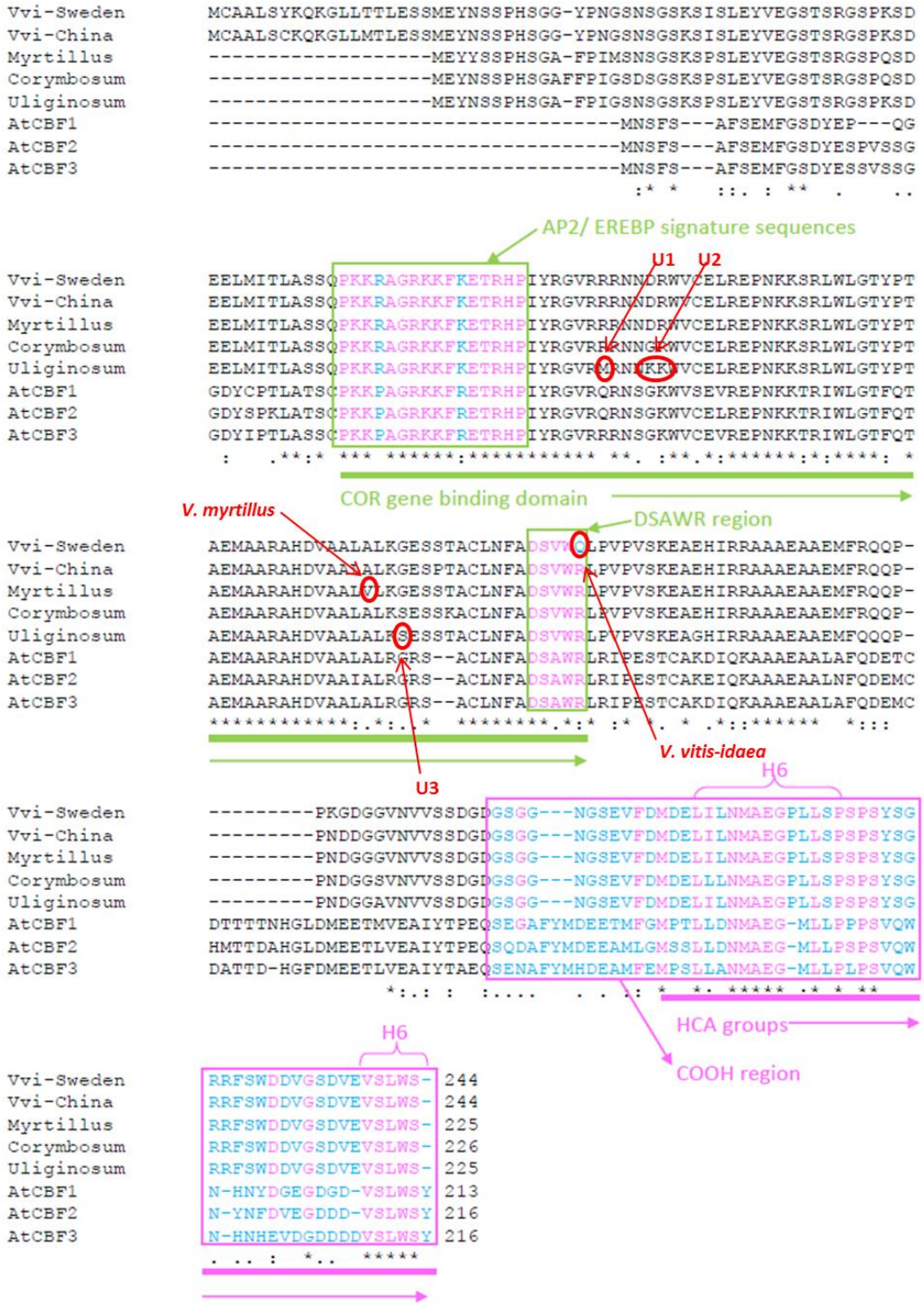

Figure 4-5: Locations of site directed mutagenesis of regions in the *COR gene* binding domain, three constructs were made for *V. uliginosum* (U1-3), one each for *V. myrtillus* and *V. vitis-idaea*.

4.2.5 Reporter gene assays for mutant GFP-VCBF/DREB1 proteins

The mutant GFP-CBF/DREB1 constructs were used for reporter gene assays in the same way as with the wild type constructs. The mutant constructs were co-infiltrated with CRT/DRE::LUC as a patch on half of a leaf, the control for each was the corresponding wild type version of each *Vaccinium CBF/DREB1* and CRT/DRE::LUC on the other half of each leaf. This was then used to work out the change in CRT/DRE activation efficiency in each case.

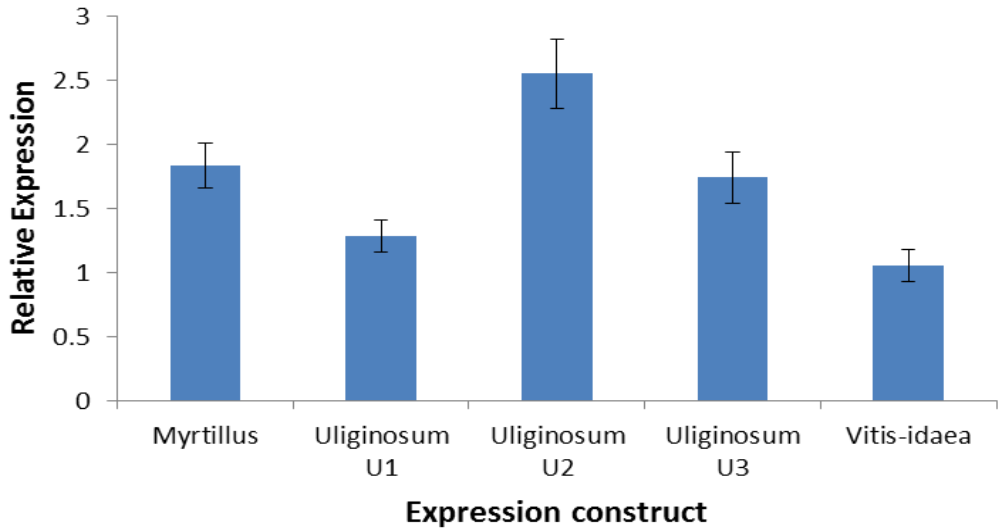

Figure 4-6: Reporter gene assays comparing CRT/DRE activation by mutated *CBF/DREB1* proteins from each species to the wild type for the corresponding species. Average luminescence for each infiltration patch was measured then divided by the luminescence from the control for 16 individual leaf replicates per construct. Error bars represent standard error of the mean.

The *V. myrtillus*, *V. uliginosum* U2 and U3 constructs have improved the expression induced by the corresponding *CBF/DREB1,* whereas the *V. uliginosum* U1 and *V. vitis-idaea* constructs showed little improvement.

4.3 Discussion

4.3.1 All three CBF/DREB1 proteins are targeted to the nucleus

The results from the GFP-CBF/DREB1 imaging show clearly that all three constructs are targeted to the nucleus. Free GFP will enter the nucleus without a nuclear localisation signal due to its size (Moore & Murphy, 2009), making the presence of the free GFP control essential. All three constructs had clear nuclear localisation and significantly less cytosolic fluorescence than the free GFP control. A better way to demonstrate that *CBF/DREB1* was constantly nuclear localised would be to use fluorescence recovery after photobleaching (FRAP) microscopy (Van Royen et al., 2009). This involves using the confocal microscope to bleach an area within the nucleus of tobacco infiltrated with each construct and measuring how long it takes to recover the fluorescence in that area and whether it returns to the original intensity (Cardarelli et al., 2012). This would show whether the GFP-CBF/DREB1 construct is constantly being transported into the nucleus.

V. uliginosum CBF/DREB1, despite showing nuclear localisation, was more difficult to image than the other constructs, as there was fainter GFP fluorescence possibly due to its stability. *V. uliginosum* has a double lysine in the middle of the *COR gene* binding domain that is not present in the other two *Vaccinium* species; this was highlighted as a potential ubiquitination site after a discussion with Dr Ari Sadanandom (Kaiser & Wohlschlegel, 2005, Pickart, 2001). The fainter GFP fluorescence in the nucleus could therefore possibly be caused by the breakdown of some of the CBF/DREB1 produced by *V. uliginosum* construct. This would release free GFP causing a fainter level of fluorescence in the nucleus and potentially higher background fluorescence. The site directed mutagenesis reporter gene assays show that it is possible that the stability of the CBF/DREB1 protein produced by the U2 expression construct was increased by the lysine to arginine mutation, which has been shown to block ubiquitination (Kaiser & Wohlschlegel, 2005). This change would allow the change in CRT/DRE activation recorded in Figure 4-6.

4.3.2 *V. uliginosum* produces altered level of GFP-CBF/DREB1

To investigate this further, Western blots were carried out from infiltrated leaf disks to look at the stability of the GFP-CBF/DREB1 protein produced. Three different time points were used to identify the optimal time to measure protein levels as there may have been variation between the three CBF/DREB1 constructs; however, both imaging and the Western blots showed the optimal time point to be 48h. It was possible to see a small amount of GFP fluorescence after 24h, after 72hours all three constructs had cytosolic

fluorescence present. At all three time points *V. uliginosum* GFP-CBF/DREB1 was almost undetectable by Western blot analysis but a strong band was present at 24kDa suggesting that free GFP was present at each time point as opposed to the GFP-CBF/DREB1 fusion protein.

The Western blots and imaging do not give any further insight into why there is a difference in CRT/DRE activation between *V. myrtillus* and *V. vitis-idaea*. Reporter gene assays were used to look further at the ability of each construct to activate CRT/DRE. The results showed that *V. myrtillus* is most effective in activating CRT/DRE; however, *V. vitis-idaea* can also activate CRT/DRE to a lower level and *V. uliginosum* to an even lesser extent. There are 5 main differences between the protein sequences of the three species which could account for the differences in CRT/DRE activation. Each of these residues was changed to match the sequence of *V. myrtillus* and the difference in CRT/DRE activation was measured. By changing the double lysine in *V. uliginosum* (U2) the activation improved significantly, possibly by improving the stability. The other mutations also improved the activation abilities of the respective *CBF/DREB1* with the exception of *V. vitis-idaea*. *V. vitis-idaea* has the sequence DSVWQ at the end of the *COR* gene binding domain whereas the other *Vaccinium* species and *Arabidopsis* have the sequence DSVWR which was identified by Canella et al., (2010) to be an essential sequence for *COR* gene binding. Changing the DSVWQ sequence present at the end of the *COR* gene binding domain from *V. vitis-idaea* by site directed mutagenesis to match the DSVWR sequence might have caused a decrease in CRT/DRE binding efficiency, instead of the hypothesised improvement.

Levels of expression of both the mutant and wt GFP-CBF/DREB1 were tested using Western blot analysis of total protein extracted from infiltrated *N. benthamiana*. This highlighted a problem in using GFP-CBF/DREB1 fusion proteins as the fusion proteins produced are approximately the same size as the large subunit of RuBisCO (~50kDa), therefore each Western blot showed false positive results with multiple different αGFP antibodies as each time there was non-specific bindingof the αGFP antibody to the large RuBisCO band present on the membrane. This was further checked using controls of both free GFP and non-infiltrated *N. benthamiana* leaves. It was possible to identify the free GFP band at 24kDa but in all samples exactly the same non-specific RuBisCO band was present. This meant that it was not possible to establish whether mutant and wt constructs were expressed or to what levels . As RuBisCO is the most abundant plant protein (Ellis, 1979, Spreitzer & Salvucci, 2002) future work to measure protein levels should include a step to remove RuBisCO from the protein extracts before starting experiments. Using a method

such as running the extract through a RuBisCO binding column (Sigma alderich Ltd, Dorset, UK) or precipitation using polyethene glycol (PEG) (Xi et al., 2006) may reduce the amount of RuBisCO present enough to measure levels of less abundant proteins such as GFP-CBF present in the extract.

4.3.3 Nuclear targeting in the mutated *Vaccinium* CBF/DREB1 proteins

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Vvi-Sweden      LASSQPKKRAGRKKFKETRHPIYRGVRRRNND
Vvi-China       LASSQPKKRAGRKKFKETRHPIYRGVRRRNND
Myrtillus       LASSQPKKRAGRKKFKETRHPIYRGVRRRNND
Corymbosum      LASSQPKKRAGRKKFKETRHPIYRGVRRRNNG
Uliginosum      LASSQPKKRAGRKKFKETRHPIYRGVRMRNNK
AtCBF1          LATSCPKKPAGRKKFRETRHPIYRGVRQRNSG
AtCBF2          LATSCPKKPAGRKKFRETRHPIYRGVRQRNSG
AtCBF3          LASSCPKKPAGRKKFRETRHPIYRGVRRRNSG
                        **    ***     *
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Figure 4-7: Amino acids important for nuclear localisation (highlighted in pink) for *Arabidopsis* CBF1-3, *V. vitis-idaea* from both China and Sweden (Vvi), *V. corymbosum* and *V. uliginosum*. The amino acids conserved between the species are marked with an asterix.

Stockinger et al., (1997) identified the nuclear localisation sequence of CBF/DREB1 within the AP2/EREBP signature sequence of *Arabidopsis* (Figure 4-7). There is one notable difference between *Vaccinium* and *Arabidopsis* where an arginine is a lysine in *Vaccinium* and is conserved in all five *Vaccinium* sequences; however, these are amino acids with similar properties, suggesting the sequence is important due to the high level of conservation between *Arabidopsis* and *Vaccinium*. Despite this difference in sequence from *Arabidopsis*, the imaging from Figure 4-1 shows that all three Arctic GFP-CBF/DREB1 fusion proteins are nuclear localised. As the site directed mutagenesis did not alter the AP2 signature sequence or the nuclear localisation sequence in any of the five mutations, the nuclear localisation of each of the five mutant GFP-CBF/DREB1 proteins produced should not have altered from the nuclear localisation shown by the *Vaccinium* wt GFP-CBF proteins.

5 Chapter 5 –Post-translational regulation of *Vaccinium* CBF/DREB1 in *Arabidopsis* and *Nicotiana benthamiana*

5.1 Introduction

CBF/DREB1 in *Arabidopsis* activates *COR* gene expression in response to cold (Gilmour et al., 1998). The combined effects of the *COR* genes in *Arabidopsis* lead to cold acclimation and freezing tolerance (Guy et al., 1985). *COR* genes encode a large number of different proteins such as heat shock proteins (HSP) (Krishna et al., 1995), fatty acid desaturases (Gibson et al., 1994), signalling pathway components such as calmodulins (Polisensky & Braam, 1996), and a large number of cryoprotective proteins (Thomashow et al., 1990). There are families of *COR* genes such as the cold inducible (KIN) genes which encode antifreeze proteins (Kurkela & Borgfranck, 1992).

The aim of the work described in Chapter 5 was to identify proteins associated *in vivo* with CBF/DREB1 from both *Arabidopsis* and *Vaccinium*. Comparisons were made between interactions with CBF/DREB1 from the three different *Vaccinium* species, and then between *Vaccinium* and *Arabidopsis*. A second aim was to identify any post-translational modification to CBF/DREB1 from any of the species.

Fusion proteins of either strep or GFP tagged CBF/DREB1 for *Arabidopsis* and the three *Vaccinium* species were produced to use in protein isolation by protein pull down. Protein was extracted from both stable *Arabidopsis* transformants and transient protein expressions in *N. benthamiana*. Pull downs were also carried out using transient expressions of the site directed mutagenesis *Vaccinium* CBF/DREB1s (Chapter 4) to compare the interactors or post-translational modifications identified by mass spectroscopy between the wt and mutant CBF/DREB1 proteins.

5.2 Results

To further characterise the post-translational regulation of CBF/DREB1 affinity-tagged CBF/DREB1 constructs were produced and expressed in *Arabidopsis* for later use in protein pull down experiments. The aim was to isolate the tagged CBF/DREB1 in order to identify any associated proteins and/ or any post-translational modifications to the CBF/DREB1 itself.

5.2.1 Characterisation of Strep-CBF/DREB1 transgenic lines: testing *CBF/DREB1* and *COR* expression

Transgenic lines of *Arabidopsis* expressing CBF/DREB1 tagged with either an N- or C-terminal Strep II tag were produced by Alex Sargeant (Knight Lab). Constructs were checked by PCR for the N-terminal strep tag-CBF/DREB1 fusion using primers designed to amplify across the strep tag and CBF/DREB1 coding sequence in both directions. A similar approach was also carried out to identify and check the C-terminal fusion constructs.

Seeds from six independent transgenic lines expressing each construct were grown for two weeks and characterised by qPCR to test expression levels (Figure 5-1). 35S N-strep ACBF /DREB1(*Arabidopsis CBF2*) lines were tested using primers designed to N-strep as the N terminal strep tag should be representative of the level of *CBF/DREB1* expression that was present. Using primers designed to unique Strep II regions to measure N-strep expression was more accurate than using primers designed to the coding sequence of *CBF2* as the four *CBF/DREB1* coding sequences in *Arabidopsis* have regions of sequence homology and primers may measure levels of more than one *CBF/DREB1*. Results from the N-strep qPCR showed a range of expression levels (Figure 5-1A) and also that, as expected, there was no expression of N-strep in the wt plants. To check that the strep-tagged CBF/DREB1 was still able to bind to and activate *COR* genes, relative levels of *KIN2* expression were measured as a representative *COR* gene (Figure 5-1B). These data showed a range of expression levels with all of the lines displaying higher *KIN2* expression than the wt control. Lines 1 and 3 were chosen as the best lines to use for further experiments due to the high levels of N-strep and *COR* gene expression.

Native promoter constructs for N-terminal Strep tagged *Arabidopsis* CBF2 were also used. Ideally it would be better to look at the associated proteins and behaviour of CBF2 under normal cold stress conditions with a native promoter rather than a 35S promoter. Constitutively expressed CBF2 may have altered protein interactions as so much more of the transcription factor is produced under a 35S promoter than even a cold induced native promoter.

Two samples for each line were tested, one under ambient conditions and one after a cold treatment to induce N-strep-CBF/DREB1 expression. Figure 5-2A shows the results of testing N-strep expression in 6 lines under both cold and ambient conditions; in each line the level of N-strep expression increased after cold treatment. Figure 5-2B shows that there was no significant increase in *COR* gene expression in response to cold in any of the transgenic lines.

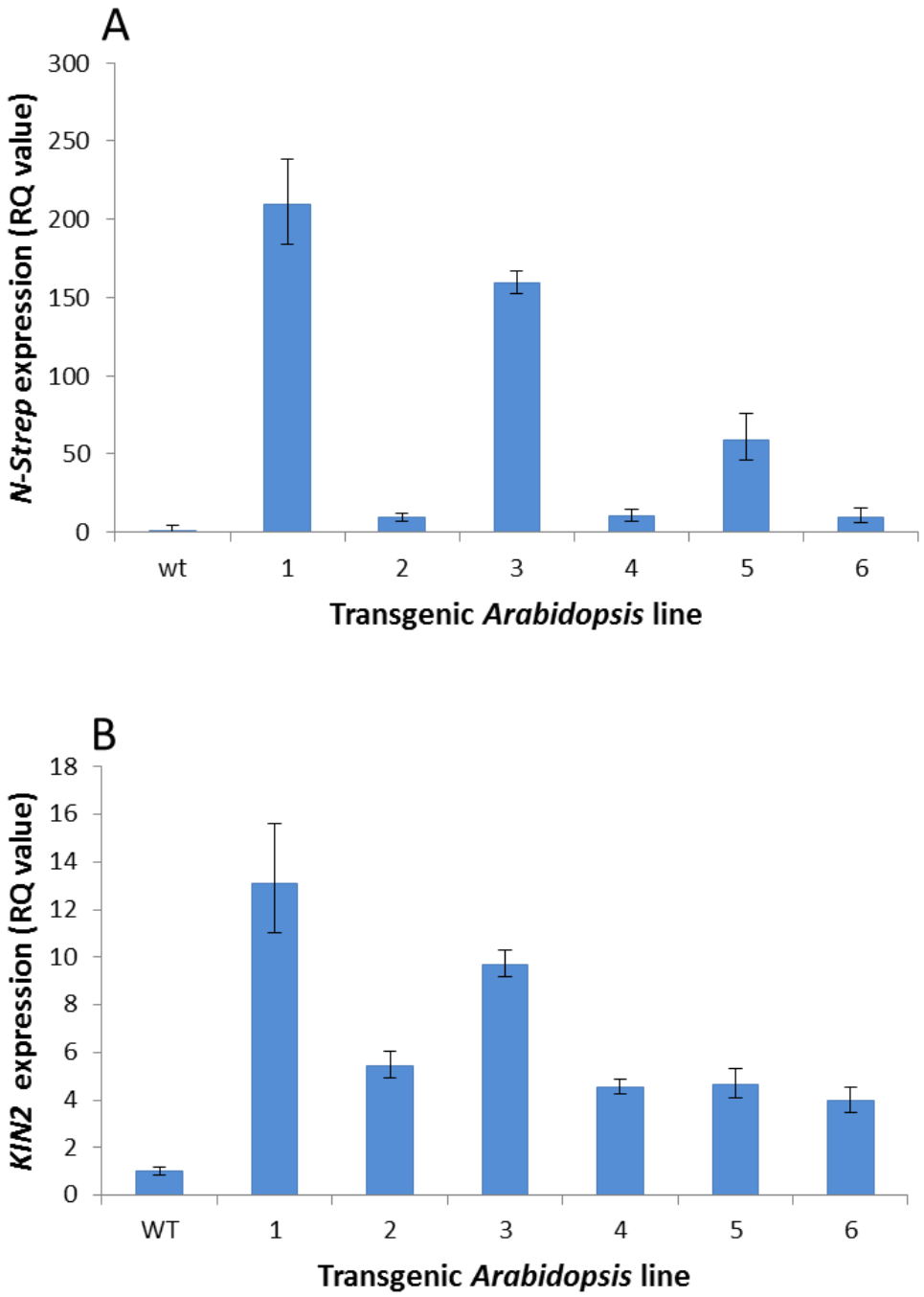


Figure 5-1: 35s N-strep *(Arabidopsis*) CBF/DREB1 lines 1-6 were tested for N-strep expression (A) by qPCR and then for *COR* gene expression using *KIN2* as a representative *COR* gene (B). Samples were normalised to the wt control sample. Error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Student's *t*-test.

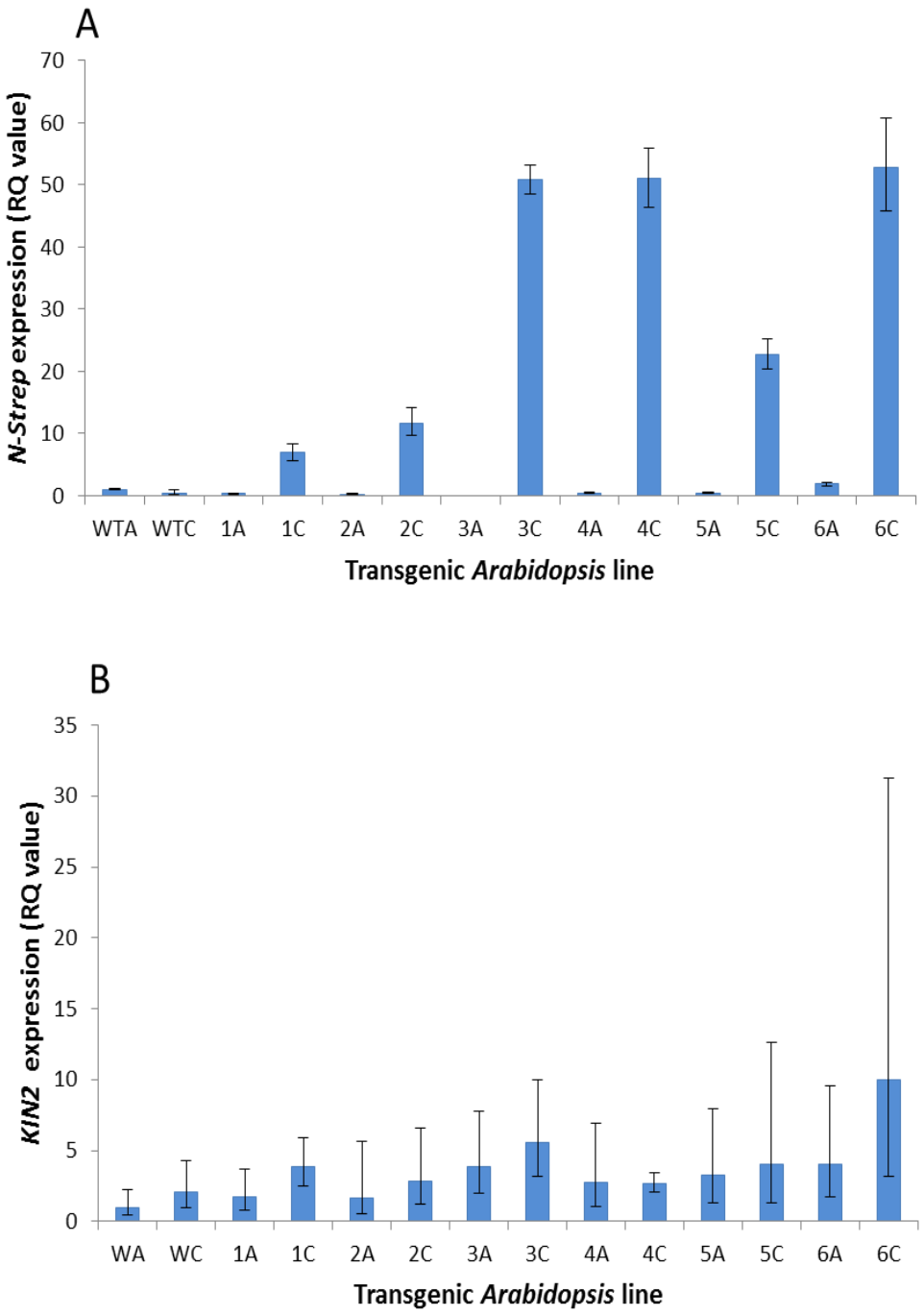


Figure 5-2: Native promoter N-terminal strep tagged *Arabidopsis* CBF constructs. Expression levels for each line were tested under both Cold (c) conditions of 4°C and ambient (a) conditions of 20°C for 6h in the dark. Both N-strep (A) and KIN2/ COR gene expression (B) were tested, with samples normalised to wt controls. Error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Student's *t*-test.

5.2.2 Isolation of Strep-tagged CBF/DREB1 and western blot analysis

The 35S N-strep-CBF2 lines 1 and 3 were chosen to be the most suitable because of the high expression of *N-strep* and *KIN2* and the native promoter lines 4 and 6 were chosen because of their high level of cold induced of *N-strep* expression.

Forty two plants of each line were grown to the rosette stage just before flowering (around 6 weeks), rosette leaves from the 35S plants were harvested and flash frozen in liquid nitrogen and the native promoter constructs were cold treated for 6h before also being harvested and flash frozen. The 35s lines produced smaller rosettes than the wt due to the over expression of CBF/DREB1; the native promoter lines were also smaller than the wt controls. As the native promoter C-strep lines showed the most severe dwarf phenotype they were not used. Because of the dwarf phenotype, the total mass of leaf tissue obtained from each individual line was too small to use; the two lines used for each promoter were therefore pooled to give one 35S sample and one native promoter sample. Total protein was extracted and run through the Strep column as described in 2.16.1. The protein extracted using the Strep column was then purified and run on a Western blot. On both occasions when this experiment was attempted there was not enough protein present to detect on the Western blot membrane with αStrep II antibody.

5.2.3 Characterisation of GFP-CBF/DREB1 transgenics

As there was insufficient protein present to detect from 84 *Arabidopsis* rosettes, new constructs were produced with a GFP tag for protein purification. A GFP tag was used as a substitute for a Strep II tag as it had previously proved more successful for protein purification from *Arabidopsis* than using strep columns.

35S GFP constructs were produced for *Arabidopsis* and with each of the three *Vaccinium* CBF/DREB1s coding sequences fused to a GFP tag. Unfortunately due to problems with black fly larvae from BASTA trays in the same growth rooms the transformants from the *V. vitis-idaea* and the *Arabidopsis* CBF/DREB1 constructs were eaten. Therefore the work with stable transformants could only be performed with *V. myrtillus* and *V. uliginosum* constructs.

Lines expressing *V. myrtillus* and *V. uliginosum* 35S::GFP-VCBF/DREB1 constructs were characterised by qPCR using primers designed to the coding sequence of *Vaccinium CBF/DREB1* to check each line was overexpressing *CBF/DREB1* and to establish whether each construct could induce an increase in *COR* gene expression as a result (Figure 5-3). Both the *V. myrtillus* and *V. uliginosum* constructs resulted in lines with a range of expression levels. These same lines were then also tested for *COR* gene expression for five

different *COR* genes. Figure 5-3 (C,D) shows both constructs were able to induce *KIN2* expression to a higher level than present in the wt Arabidopsis. For both constructs the lines expressing the highest levels of *CBF/DREB1* did not induce the highest level of *KIN2* expression.

Figure 5-4 also shows that both constructs were able to induce *COR* gene expression. Part A/B shows that the pattern of *GOLS3* expression differs from *KIN2* as the *V. myrtillus* lines with high *CBF/DREB1* expression induce the highest expression of *GOLS3*. However, the *GOLS3* expression pattern for the *V. uliginosum* lines is the same as the expression for *KIN2,* in that the lines with the highest expression of *CBF/DREB1* do not induce the highest levels of *GOLS3* expression. However, the *V. myrtillus* lines were able to induce a much greater increase in expression of *GOLS3* than the *V. uliginosum* lines.

Figure 5-4 (C/D) shows that *V. myrtillus* could also induce a greater level of *LTI78* expression than *V. uliginosum* lines, again with a similar expression pattern to *KIN2,* in that the lines with the highest expression of *CBF/DREB1* have the lowest expression of *LTI78*. The *V. uliginosum* results also show this same pattern.

In Figure 5-5 (A/B) the *V. myrtillus* lines display a much greater increase in *COR414* expression than the *V. uliginosum* construct (~80 times higher than wt in comparison to ~1.5 times higher than wt). In contrast to *KIN2* and *LTI78* expression the highest level of *COR414* in the *V. myrtillus* lines was induced by the lines expressing the highest levels of *CBF/DREB1*. However, following this pattern transgenic line M5 should show *COR414* induction which it doesn't. The *V. uliginosum* lines have induced *COR414* expression in the same pattern as *KIN2*, *GOLS3* and *LTI78,* in that the lines expressing lower levels of *CBF/DREB1* have the highest expression of *COR414.* However, the *V. uliginosum* lines could not cause *COR414* induction to levels much higher than the wt *Arabidopsis*.

Again for *COR15A* (Figure 5-5 C/D) the highest expression was induced by the lines expressing the lowest levels of *CBF/DREB1* for both the *V. uliginosum* and *V. myrtillus* lines, with both lines showing the highest expression of *COR15A* to almost exactly the same level of expression, (greatest increase for *V. myrtillus* is x12 and for *V. uliginosum* x11.5). The *V. myrtillus* line expressing the highest levels of expression of *CBF/DREB1* could not induce *COR15A* expression to a higher level than the wt *Arabidopsis*.

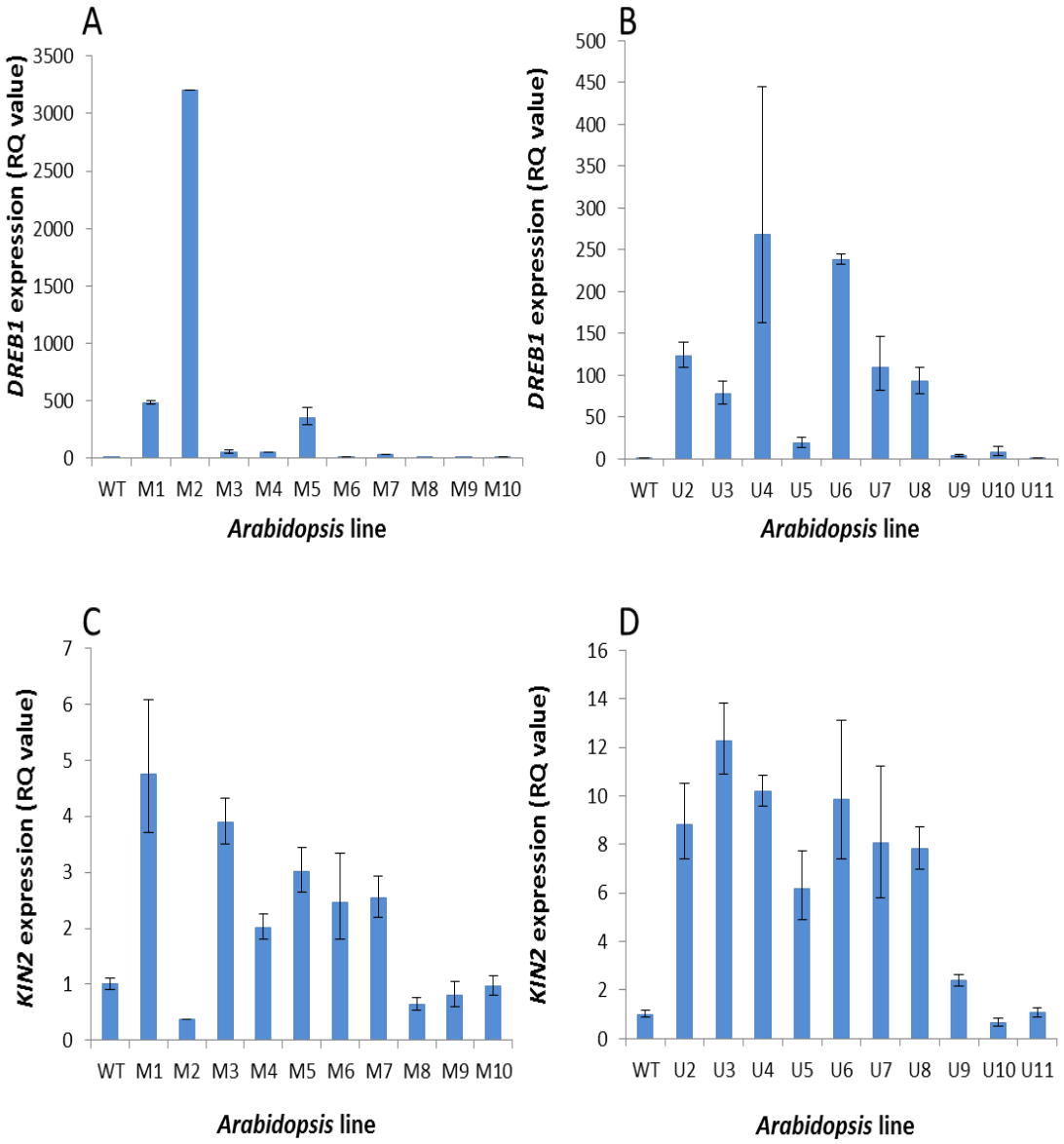


Figure5-3: Ten independent transgenic lines of *Arabidopsis* GFP-VCBF/DREB1 seedlings were tested for *CBF*/*DREB1* (A,B) and *KIN2* (C,D) expression levels by qPCR. For lines containing *V. uliginosum* CBF (U) and *V. myrtillus* CBF (M), samples were normalised to the wt control. Error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Student's *t*-test.

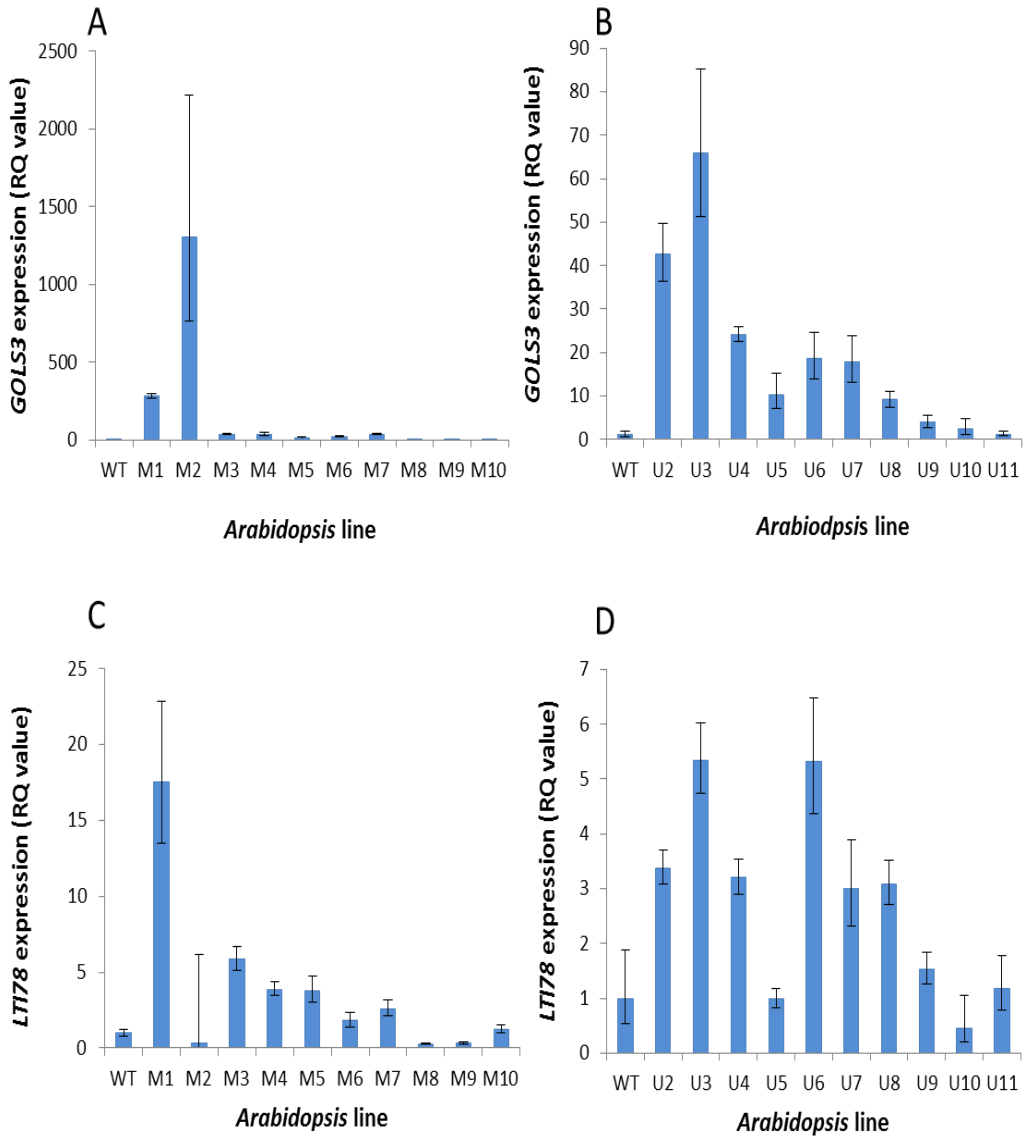


Figure 5-4: Ten independent transgenic lines of *Arabidopsis* GFP-VCBF/DREB1 were then tested for *GOLS3* (A,B) and *LTI78* (C,D) expression. For lines containing *V. myrtillus* CBF (M) and *V. uliginosum* CBF (U), samples were normalised to the wt control. Error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Student's *t*-test.

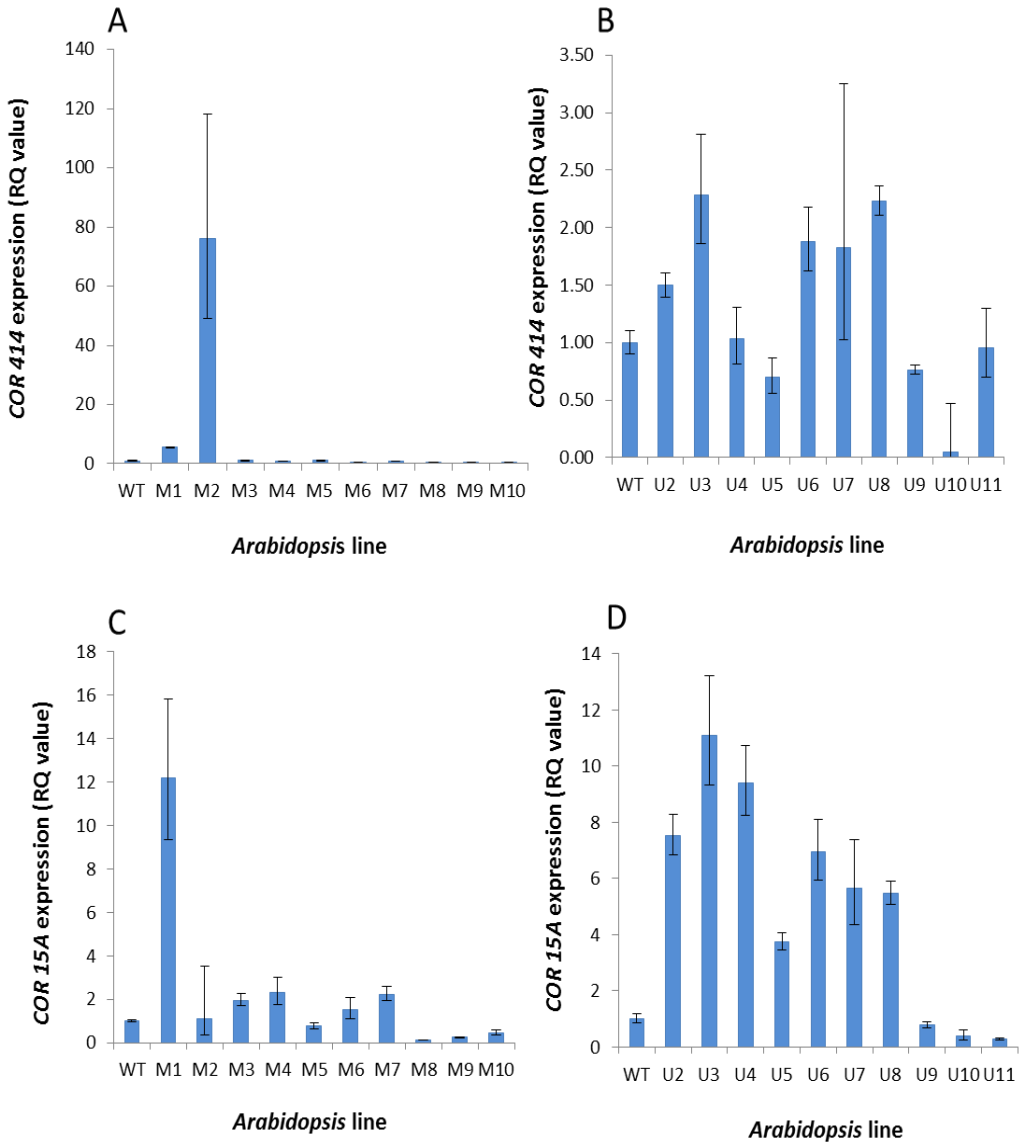


Figure 5-5: Ten independent transgenic lines of *Arabidopsis* GFP-VCBF/DREB1 lines were tested, for *COR414* (A,B) and *COR15A* (C,D) expression. For lines containing *V. myrtillus* CBF (M) and *V. uliginosum* CBF (U), samples were normalised to the wt control. Error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Student's *t*-test.

5.2.4 Pull-downs of GFP-CBF/DREB1 fusion proteins

The stable transformants expressing 35S::GFP-VCBF/DREB1 displayed a similar dwarf phenotype to those overexpressing constructs without a GFP tag as described in chapters 3 and 4. Because of this it took a long time to grow each generation of *Arabidopsis*. This was also combined with problems with fungus gnat larvae originating from BASTA trays in the same growth rooms as the overexpression lines.

The first pull downs were carried out using lines expressing the 35S::GFP-VCBF/DREB1 constructs produced by site directed mutagenesis with the wt *Vaccinium* CBF/DREB1 GFP tagged constructs as controls, all expressed transiently in *N. benthamiana* (following the method described in 2.14.2.3). Controls of a non-infiltrated leaf and leaf tissue expressing free GFP were also used. This allowed any non-specific proteins that were collected with the αGFP beads to be easily identified. The extracts were run on an SDS PAGE gel and protein bands that appeared different between the lanes after silver staining were highlighted for identification by Mass Spectroscopy.

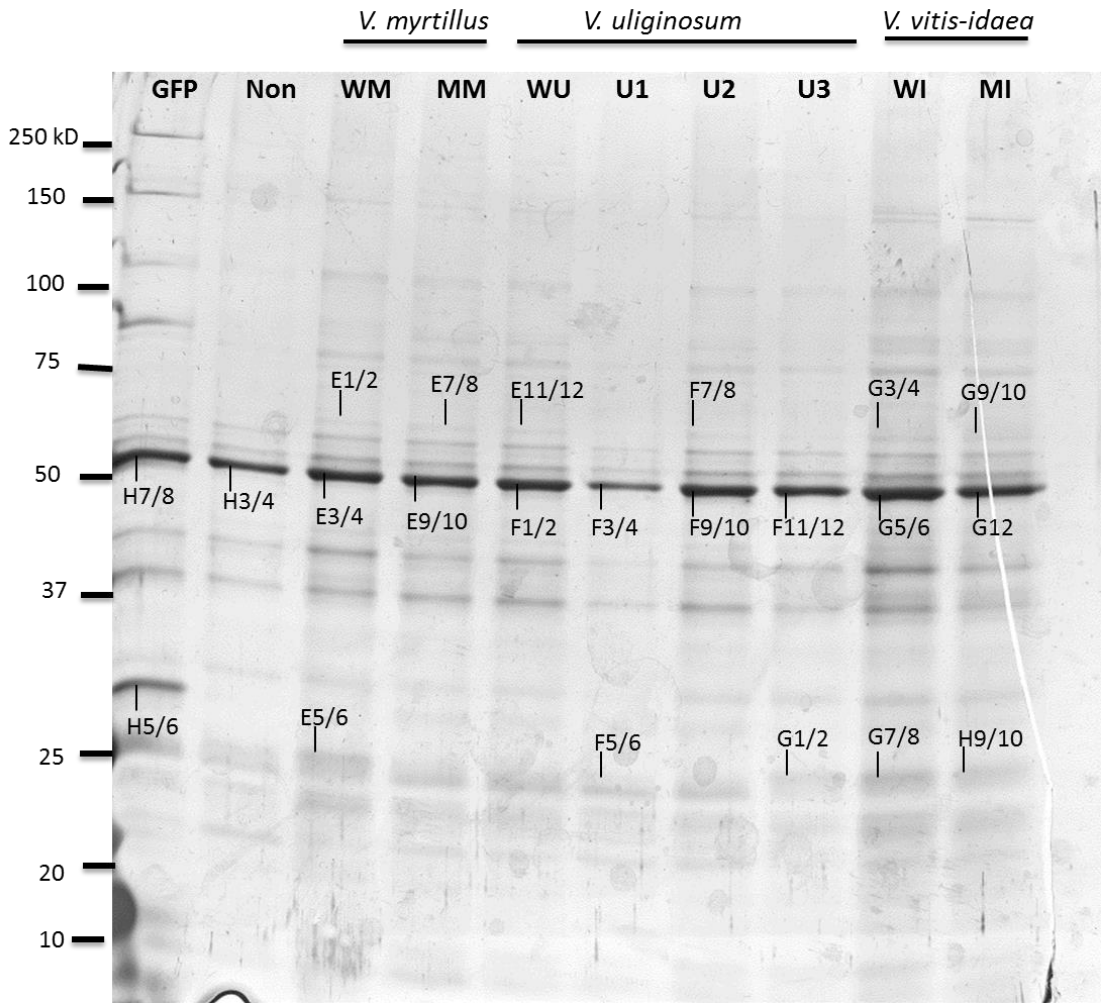


Figure 5-6: GFP pull downs from transiently expressed constructs for wild type *V. myrtillus* (WM), *V. uliginosum* (WU) and *V. vitis-idaea* (WI) constructs of 35S::GFP-VCBF and mutant constructs (as described in chapter 4) of *V. myrtillus* (MM), *V. uliginosum* mutations 1, 2, 3 (U1, U2, U3) and *V. vitis-idaea* (MI). Controls of free GFP (GFP and non infiltrated leaf (Non) were used. Numbered bands were isolated for mass spectrometry, numbers correspond to Table 1.

As there was a strong band present around 50kDa in the control samples (Figure 5-6) around the same size GFP-CBF should be found. It was possible that the large subunit of RuBisCO was present in all of the samples which would make it difficult to identify other proteins present by mass spec as RuBisCO is present in such large quantities. To avoid this problem a second pull down was carried out using a stable 35S::GFP-VCBF/DREB1 *V. uliginosum Arabidopsis* line and wt *Arabidopsis.* Both were grown entirely in the dark on MS plates for 7 days to avoid the production of RuBisCO (Figure 5-7). The protein extractions, pull downs and analysis were then carried out in the same way as for the infiltrated samples.

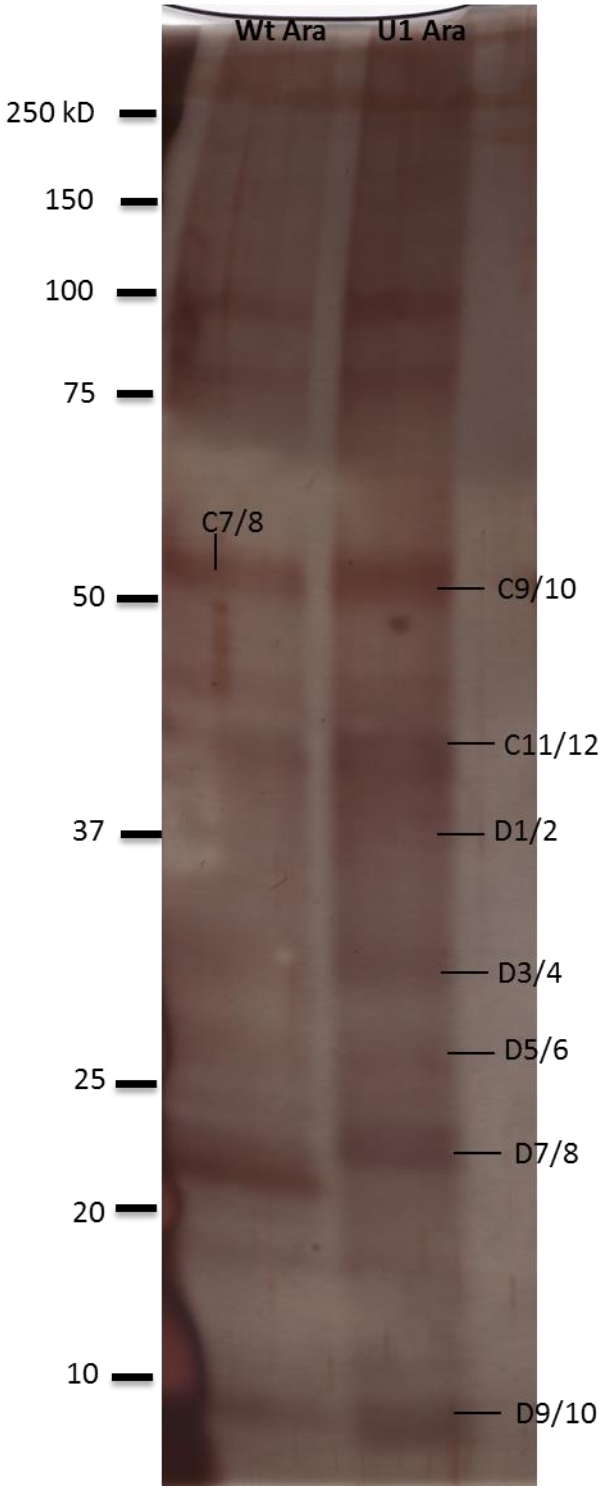


Figure 5-7: GFP pull downs from wt *Arabidopsis* (Wt ara) and *Arabidopsis* expressing *V. uliginosum* 35S::GFP-VCBF/DREB1 (U1 Ara) both grown in the dark. Numbered bands were isolated for mass spectrometry. Numbers correspond to Table 1.

5.2.5 Mass spectrometry

The bands labelled in figures 5-6 and 5-7 were processed by Durham University Proteomics Facility. Protein bands were cut out and trypsin digested using a Progest automated digester (Genomic solutions Ltd, Bath, UK) before running on a QSTAR pulsar I Q-Tof mass spectrometer (AB Sciex Ltd, Cheshire, UK).

As there were not large amounts of protein present in some of the bands, each band was sampled twice and the extracted, digested protein was then pooled for analysis, (hence most mass spectrometry samples consisting of two numbers). The ion fragment data were analysed using the SwissProt plant database (Boeckmann, Blatter, Famiglietti, Hinz, Lane, Roechert & Bairoch, 2005, Schneider, Lane, Boutet, Lieberherr, Tognolli, Bougueleret & Baiyoch, 2009). The genome sequence of *Nicotiana benthamiana* has not been fully assembled and annotated so the results could only be in the form of the next nearest match to the proteins identified from the proteins pulled down from *N. benthamiana*.

The majority of protein samples identified were the large subunits of RuBisCO (see Table 5-5). To identify any proteins present in the same band but at lower abundance, the protein sequence and theoretical digest sequences for eGFP and the three *Vaccinium* CBF/DREB1s were added to the ProteinPilot™ software (AB SCIEX, Warrington, Cheshire, UK) (Appendix E –Theoretical digests for mass spectrometry). By searching the results again with the instruction to ignore RuBisCO, eGFP was identified from band H5/6 (figure 5-6) which hadn't previously been identified in the first search. Other chloroplastic proteins were also identified but it was not possible to detect any of the *Vaccinium* sequences in the proteins extracted. Unfortunately the protein bands from Figure 5-7 did not contain enough protein to identify any of the proteins present by mass spectroscopy.

Table 6.5: Proteins in each band extracted from the gels in figures 6 and 7, identified by mass spectroscopy analysis. Blue highlighted areas represent control bands for Figure 6 with the white area containing the experimental bands for Figure 6. Pink area contains the results from *Arabidopsis* protein bands extracted from the gel in Figure 6.7.

Band	Acession number	Protein
H5/H6	SP Z0001	VAC10_VACMY GFP Ghent vectors
H7/H8	SP Q8RU60	RBL_ATRBE Ribulose biphosphate carboxylase large chain OS- *Atropa belladonna*
H3/4	SP Q8RU60	RBL_ATRBE Ribulose biphosphate carboxylase large chain OS- *Atropa belladonna*
E1/2	SP P21239	RUB1_BRANA RuBisCO large subunit-binding protein subunit alpha, chloroplastic (Fragment) OS- *Brassica napus*
E3/4	SP Q3C1J4	RBL_NICSY Ribulose bisphosphate carboxylase large chain OS-*Nicotiana sylvestris*
E5/6	SP P27141	CAHC_TOBAC Carbonic anhydrase, chloroplastic OS- *nicotiana tabacum*
E7/8	SP P21241	RUBB_BRANA RuBisCO large subunit-binding protein subunit beta, chloroplastic OS-*Brassica napus*
E9/10	SP Q8RU60	RBL_ATRBE Ribulose biphosphate carboxylase large chain OS- *Atropa belladonna*
E11/12	SP Q42694	RUBA_CHLRE RuBisCO large subunit-binding protein subunit alpha, chloroplastic OS- *Chlamydomonas reinhardtii*
F1/2	SP Q3C1J4	RBL_NICSY Ribulose bisphosphate carboxylase large chain OS-*nicotiana sylvestris*
F3/4	SP Q8RU60	RBL_ATRBE Ribulose biphosphate carboxylase large chain OS- *Atropa belladonna*
F5/6	SP P27141	CAHC_TOBAC Carbonic anhydrase, chloroplastic OS- *nicotiana tabacum*
F7/8	SP Q9LJE4	CPNB2_ARATH Chaperonin 60 subunit beta 2, chloroplastic OS-*Arabidopsis thaliana*
F9/10	SP Q3C1J4	RBL_NICSY Ribulose bisphosphate carboxylase large chain OS-*nicotiana sylvestris*
F11/12	SP Q3C1J4	RBL_NICSY Ribulose bisphosphate carboxylase large chain OS-*nicotiana sylvestris*
G1/2	SP P27141	CAHC_TOBAC Carbonic anhydrase, chloroplastic OS- *nicotiana tabacum*
G3/4	SP P08824	RUBA_RICCO RuBisCO large subunit-binding protein subunit alpha (fragment) OS- *Ricinus communis*
G5/6	SP Q3C1J4	RBL_NICSY Ribulose bisphosphate carboxylase large chain OS-*nicotiana sylvestris*
G7/8	SP P27141	CAHC_TOBAC Carbonic anhydrase, chloroplastic OS- *nicotiana tabacum*
G9/10	SP B1NWD5	ATPA_MANES ATP synthase subunit alpha, chloroplastic OC- *Manihot esculenta*
G12	SP Q8RU60	RBL_ATRBE Ribulose biphosphate carboxylase large chain OS- *Atropa belladonna*
H9/10	SP P27141	CAHC_TOBAC Carbonic anhydrase, chloroplastic OS- *nicotiana tabacum*
C7/8	SP 003042	RBL_ARATH Ribulose bisphosphate carboxylase large chain OS- *Arabidopsis thaliana*
C9/10	SP 003042	RBL_ARATH Ribulose bisphosphate carboxylase large chain OS- *Arabidopsis thaliana*
C11 to D11	No proteins present	Not enough protein present to identify

5.3 Discussion

5.3.1 Strep-tagged fusion proteins vs GFP fusion proteins

Strep II tagged protein pull downs were carried out using both native promoter and 35S promoter lines for protein extractions and analysis, the strep tag pull downs did not have enough protein to detect even using 84 *Arabidopsis* rosettes, with either the native promoter lines after cold treatment or a 35S promoter lines but for both lines no fusion protein was detectable. The GFP tagged lines were made as an alternative to the strep tagged lines, as it wasn't clear at this point why the Strep II tagged pull downs wouldn't work for *Arabidopsis* and the *Vaccinium* CBF/DREB1s would be potentially more difficult to isolate. Adding a GFP tag would potentially stabilise any of the CBF/DREB1s that were unstable and allow them to be isolated with the added bonus that a GFP tag allows the fusion protein produced to be imaged too.

A disadvantage of the CBF/DREB1 overexpression lines was that the seeds took around 2 weeks to germinate (which is much slower than wt *Arabidopsis*); this often meant that the MS plates had begun to go mouldy before all of the overexpression line seeds had germinated. The seedlings growing in mouldy patches appear to have been able to grow successfully on kanamycin plates regardless of whether they were transformants or wt therefore all appearing as though they were transformants containing the kanamycin resistance gene. This meant that testing all of the identified transformants for *Vaccinium CBF/DREB1* expression often produced false positives as well as characterising the expression levels of successful transformants. The genuine 35s::GFP-VCBF/DREB1 expressing plants grew very slowly as did the 35S-VCBF/DREB1 plants as described in chapter 3. The *V. vitis-idaea* and *Arabidopsis* GFP-CBF/DREB1 lines once identified and transferred to peat plugs were infested by fungus gnat larvae, so subsequent experiments had to continue without these two lines.

5.3.2 GFP-CBF/DREB1 fusion proteins have altered *COR* gene induction

When the *V. uliginosum* and *V. myrtillus* lines were tested for *CBF/DREB1* expression the results appeared to be similar to the results from the 35S::CBF/DREB1 lines in Chapter 3. Although the *CBF/DREB1* expression results appeared similar to the 35S::CBF/DREB1 lines the *COR* gene expression was different for the 35S::GFP-VCBF/DREB1 lines tested. This made any conclusions drawn from these plants questionable especially as the *V. myrtillus* GFP-CBF/DREB1 lines now had an altered pattern of *COR* gene expression. In Figure 5-3A the line M2 has a high level of *CBF/DREB1* expression but a variable ability to induce an

increased level of *COR* gene expression (*KIN2* (Figure 5-3C), *LTI78* (Figure 5-4C) and *COR15A* (Figure 5-5C)). If the CBF/DREB1 from *V. myrtillus* has the ability to cause variable induction of *COR gene* expression, then the 35S::CBF/DREB1 lines in Chapter 3 should have shown the same variable induction of *COR* genes. GFP is a large protein tag (26.9kDa) and essentially doubles the size of the CBF/DREB1 protein produced, possibly altering the shape of the CBF/DREB1 produced, therefore altering its ability to induce *COR* gene expression. However, when the *V. myrtillus* GFP-CBF was transiently expressed it could induce expression via the CRT/DRE element (chapter 4). To further characterise whether the GFP tag is altering the ability of *V. myrtillus* CBF/DREB1 to bind or activate *COR* genes, a non-tagged CBF/DREB1 construct would also need to be used for reporter gene assays for comparison. It would also be interesting to look at *COR* gene expression levels in an *Arabidopsis* line expressing free GFP, to establish whether GFP itself can induce *COR* gene expression. There have been reported examples in humans where GFP itself can activate gene expression (Zhang, Hackett, Lam, Cheng, Pergolizzi, Luo, Shmelkov, Edelberg, Crystal & Rafii, 2003), there have also been examples in plants where GFP tags have been added to prevent genes of interest being silenced. It is therefore possible that by adding a GFP tag the activity of CBF/DREB1 was altered so it cannot be used as a true comparison to what would occur under normal conditions.

The *V. uliginosum* 35S::GFP-CBF/DREB1 lines were able to activate *COR* gene expression with the same expression pattern for all five *COR* genes tested. This result mirrors the results from the *V. myrtillus* lines in Chapter 3. However, the results from imaging the 35S::GFP-VCBF *V. uliginosum* lines and the Western blots in Chapter 4 suggest that the *V. uliginosum* CBF/DREB1 has reduced stability in comparison to the other two *Vaccinium* species. It is possible that addition of a GFP tag has stabilised the *V. uliginosum* CBF/DREB1 allowing an increased ability to bind and activate *COR* gene expression in *Arabidopsis*. This was also suggested by the appearance of a dwarf phenotype in the *V. uliginosum Arabidopsis* GFP::VCBF/DREB1 lines. The appearance of a dwarf phenotype in the GFP-CBF/DREB1 fusion version of *V. uliginosum* CBF/DREB1 suggests that the GFP has changed the stability or activity of the CBF/DREB1 as it now causing an inhibition of GA expression, due to an increase in DELLA proteins (Fleet & Sun, 2005). This inhibition of GA expression indicates that the addition of the GFP tag to the CBF/DREB1 from *V. uliginosum* allows the induction of a stress response in the transgenic *Arabidopsis* lines produced. To test whether the ability to bind *COR* genes in the 35S::GFP-VCBF/DREB1 lines from *V. uliginosum* was due to the GFP stabilising the CBF/DREB1 in the fusion protein produced, the reporter gene

assays would need to be repeated comparing the *COR* gene binding and activation (therefore the luciferase luminescence produced) between the *V. uliginosum* 35S::GFP-VCBF/DREB1 construct and the 35S-VCBF/DREB1 construct produced (as described in Chapter 3).

As all of the *COR* genes tested are cold induced there should be no difference in *COR* gene activation/expression for all five *COR* genes induced by the GFP-CBF/DREB1 protein from *V. myrtillus.* Lines showing the lowest level of *CBF/DREB1* expression displayed the highest level of *COR* gene expression, suggesting that it is possible there is a threshold level of *CBF/DREB1* expression above which *COR* gene expression is inhibited.

5.3.3 Pull-downs of GFP fusion proteins

The GFP pull downs from infiltrated *N. benthamiana* had the large subunit of RuBisCO present to levels that made detecting less abundant proteins almost impossible and there have been examples of RuBisCO binding with αGFP antibody coated beads. If subunits of RuBisCO are able to bind the αGFP beads, as RuBisCO is so abundant the beads may not have bound much of the GFP tagged protein as the beads have a limited amount of antibody and therefore binding capacity and it may have been mostly taken up binding to RuBisco. As RuBisco is difficult to remove from extracted protein without damaging other proteins in the extract the *Arabidopsis* seedlings used for the next pull down were grown in the dark to reduce the amount of RuBisCO production. However, this also significantly reduced the amount of tissue produced from which to extract protein and uses a large quantity of seeds for a small amount of tissue. The amounts of protein present in the *Arabidopsis* pull downs was therefore not high enough to detect by mass spectrometry.

To avoid some of the problems it would be interesting to try a second pull down with the supernatant removed from the first pull down. If the RuBisCO is saturating the GFP antibody on the beads it will remove a large quantity of the RuBisCO present. This would then leave a large quantity of GFP tagged protein in the supernatant that is usually discarded, incubating this supernatant with GFP beads a second time may result in more GFP tagged protein being isolated. It may in future experiments be better to switch to magnetic beads as the number of manufacturers is higher than agarose beads. This would allow several different manufacturers to be tested and the most specific antibody to be selected to reduce background.

The numbers of washes were dictated by a compromise between washing the beads enough to remove non-specific protein and not washing so much that the associated protein or a lot of beads were lost. Possibly increasing the number of washes of the beads

at the final step would allow any aggregates of unwanted protein formed to be removed. This would reduce the amount of non-specific unwanted bands present as background when the samples were run on an SDS PAGE gel.

Optimising the method to improve binding specificity and reduce collection of unwanted proteins would be preferable to using more tissue for these experiments, especially the infiltrations, as by increasing the amount of tissue used it would further increase the relative amount of RuBisCO present.

As the GFP tag has potentially altered the activity of the CBF/DREB1 protein produced and has resulted in a fusion protein about the same size as the large subunit of RuBisCO, if this was to be repeated a better alternative would be to use a smaller protein tag such as an HA or HIS tag which can still be used for purification but that are not so big they will result in a fusion protein the same size as the large subunit of RuBisCO. A smaller tag also may not interfere with the activity of the proteins it is fused to; however, as the Strep-tagged fusion pull-downs did not work it is possible that other small tags may not work either.

Improvements in the stability of *V. uliginosum* CBF/DREB1s were suggested by the improved *COR* gene induction in the stable transgenic lines of 35S::GFP-VCBF/DREB1 when compared to the same *COR* gene expression studies carried out with 35S-VCBF/DREB1 lines described in Chapter 3. If the stability of the CBF/DREB1s was improved by adding a GFP tag and it is still not possible to isolate CBF/DREB1 it is possible that CBF/DREB1 is still not stable. It was possible to see the fusion protein under a microscope so it is not that there was a low abundance of protein. However, as GFP is nuclear localised like the CBF/DREB1 fusion protein it is possible that some of the nuclear GFP observed was free GFP as opposed to tagged protein (Moore & Murphy, 2009). This would also suggest that changing the affinity tag to allow improved technical methods may again reduce stability of the protein therefore making successful isolation of CBF/DREB1 less likely.

6 Chapter 6 – identification and characterisation of *Vaccinium* genes involved in freezing tolerance

6.1 Introduction

The three species of *Vaccinium*, *V. myrtillus*, *V. uliginosum* and *V. vitis-idaea* are dwarf shrubs found growing in the same regions of the sub-Arctic. However, each species has a slightly different physiology and growth area which is thought to lead to differences in the level of freezing tolerance each species can achieve. *V. myrtillus* is a green stemmed deciduous chamaephyte (Ritchie, 1956) which is thought to have the lowest level of freezing tolerance due to its need for relatively sheltered growth environments. *V. uliginosum* is woody stemmed, deciduous (Jacquemart, 1996) and is frequently found growing in exposed areas suggesting *V. uliginosum* has a high level of freezing tolerance. *V. vitis-idaea* is evergreen (Ritchie, 1955) and can be found growing in both exposed and sheltered areas, suggesting it is more freezing tolerant than *V. myrtillus*.

Chapters 3 to 5 were based on working with the *CBF/DREB1* sequence from the three *Vaccinium* species and what affect they could induce in the same genetic background (*Arabidopsis*). Chapter 6 aimed to establish what the *CBF/DREB1* response of each *Vaccinium* species was in response to cold, comparing both levels of expression and the time taken to induce a response between each of the three *Vaccinium* species.

Much of the CBF/DREB1 pathway has been characterised in *Arabidopsis* (Figure 6-4). Inducer of CBF expression 1/2 (ICE1/2) are transcription factors upstream of CBF/DREB1 that are constitutively expressed (Chinnusamy et al., 2003a). Under ambient conditions high expression of osmotically responsive gene 1 (HOS1) negatively regulates ICE1 by targeting ICE1 for degradation (Dong et al., 2006). Under cold conditions, small ubiquitin like modifier E3 ligase (SIZ1) sumoylates ICE1 allowing ICE1 to activate expression of *CBF/DREB1* (Miura et al., 2007). Sumoylated ICE1 also negatively regulates myeloblastosis transcription factor 15 (*MYB15*) expression in response to cold (Miura & Hasegawa, 2008). Under ambient conditions, MYB15 negatively regulates CBF/DREB1 expression (Agarwal et al., 2006).

Eskimo (ESK1) and high expression of osmotically responsive gene 9 (HOS9) are CBF/DREB1 independent and are thought also to play a role in freezing tolerance in *Arabidopsis* (Van Buskirk & Thomashow, 2006, Xin & Browse, 1998).

A second question, investigated in Chapter 6 was to determine whether there are differences in expression of other components of the CBF/DREB1 pathway and non-CBF/DREB1 pathways between the three *Vaccinium* species? This was carried out by isolating and sequencing *ICE*, *MYB15*, *ESK1*, *HOS1*, *HOS9* and *SIZ1*. The newly sequenced genes were used to design qPCR primers, which could then be used to measure expression of each of these genes in response to cold alongside *CBF/DREB1*. To build a more detailed pattern of gene expression in each *Vaccinium* species in response to cold.

6.2 Time course experiments

The aim of the *Vaccinium* time course experiments was originally to establish whether the three *Vaccinium* species express CBF/DREB1 in response to cold and if there was a difference in the expression levels and timing between the three species.

As the time course experiments were carried out over a period of three years with each experiment acting as an optimisation of methods for the following year. As such, the methods and results are described below.

6.2.1 2010 preliminary *Vaccinium* cold treatment methods

In a preliminary experiment, samples of *V. myrtillus*, *V. uliginosum* and *V. vitis-idaea* were sourced from Abisko, Sweden, wrapped in tin foil, cold-treated on wet ice for 2h or left at ambient temperature on the bench. Total RNA was extracted and cDNA synthesised for qPCR. Levels of CBF/ DREB1 expression were measured using qPCR with primers designed to be 100% homologous to the CBF/DREB1s of all three species so that the results were comparable between species. As the regions of sequence chosen to design primers to were identical between the three species the primers had the same efficiency of hybridisation in each species allowing direct quantitative comparisons to be made between CBF/DREB1 expression in the three species.

6.2.2 2010 preliminary *Vaccinium* cold treatment results

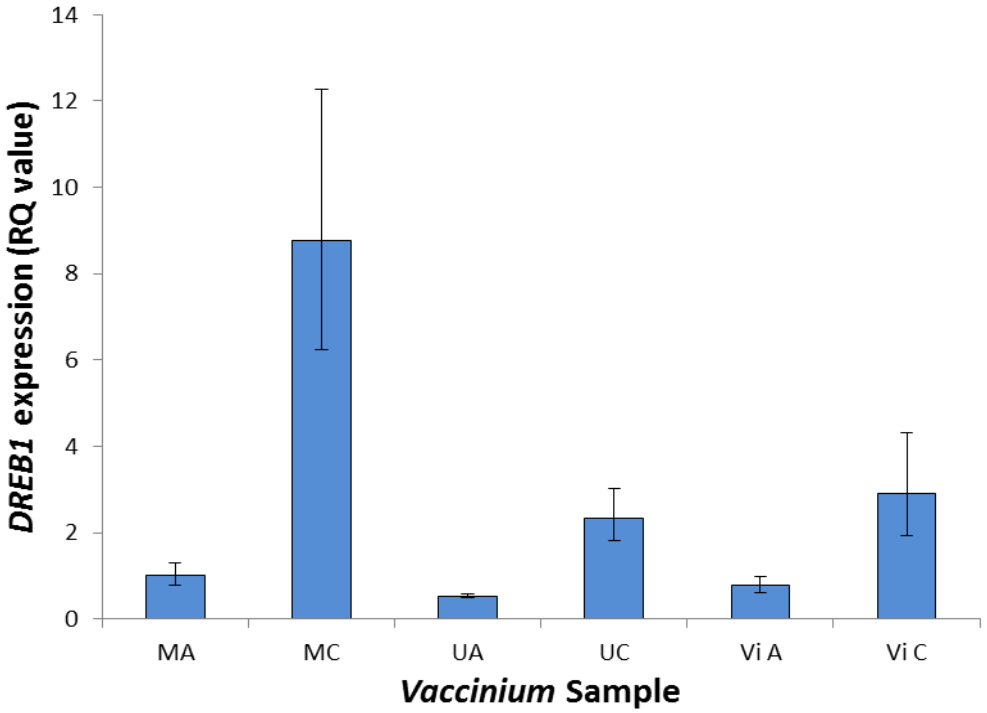


Figure 6-1: qPCR results for *Vaccinium* DREB1/ CBF expression after 2 hours cold treatment (C) on ice, or ambient conditions (A) of 20°C. For *V. myrtillus* (M), *V. uliginosum* (U) and *V. vitis-idaea* (Vi). Each cold sample is normalised to its own ambient sample. Error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Student's *t*-test.

The results in Figure 6-1 show that after 2h on ice all three species display an increase in *DREB1/ CBF* expression in response to cold. *V. myrtillus* has the greatest increase in *DREB1* expression with *V. uliginosum* and *V. vitis-idaea* displaying a relatively small increase in response to the same cold treatment. The results show that all three species have a *CBF/DREB1* response to a sudden cold shock. However, using only one time point, it is impossible to know whether the expression level obtained for each species is the maximal level of *CBF/DREB1* expression or whether it is the beginning or end of an increase in expression. An extended time course was therefore needed to establish detailed information regarding the *CBF/DREB1* response to cold in each species.

6.2.3 2011 *Vaccinium* cold stress time course method.

After testing the 2010 cold treatment samples it was clear that this experiment needed to be repeated as a time course to include a broad range of time points, this would then include any *CBF/DREB1* expression that had potentially been missed by only looking at 2h.

Vaccinium plants were dug up from Abisko (68°, 19'35.5 N, 18°, 49'57.0 E, Elevation 525m asl) with as much root tissue included as possible in mid-August 2011. Plants were then very carefully root washed and stored outside overnight in water before being wrapped in damp tissue and stored in sealed tupaware containers to return to the UK. Conditions in two identical fitotron Arctic chambers (Weiss Gallenkamp, Loughborough, UK) were pre-set to match the weather conditions in the Arctic when the plants were collected (conditions used are listed in Table 6.6). The plant samples for each species were divided into two sets, each with their roots fully submerged in water contained in glass beakers. One beaker for each species was put into each of the two growth chambers, Each chamber had samples of *V. myrtillus, V. uliginosum* and *V. vitis-idaea*). The plants were left to recover for 3 days before the temperature in one of the chambers was lowered to 1°C with all other conditions remaining identical in both chambers. Samples were taken from each plant in both chambers at time points 0a*, 0b*, 1h, 2h, 3h, 4.5h, 6h, 12h, 24h, 48h and 72h. Each sample of leaves and stem was put into a separate 50ml plastic tube and flash frozen in liquid nitrogen before being stored at -80°C for later RNA extraction.

*The time points 0a and 0b were both recorded as the chamber takes 1h to drop in temperature from ambient to cold. Therefore 0a is the true starting point of the experiment as the temperature begins to drop and 0b is the true starting point of the temperature at a constant of 1°C.

Table 6.6: Programmes run in the two Arctic growth chambers for cold treating *Vaccinium* in the 2011 time course. Light conditions and temperatures in the two chambers were set to match the fluctuations in temperature and day length in Abisko when the plants were collected as closely as possible.

Arctic chamber set conditions for weather matching (ambient) and cold treatment of *Vaccinium*							
Ambient				Cold			
Time	**Temperature**	**Humidity**	**Light**	**Time**	**Temperature**	**Humidity**	**Light**
12:00	15	80%	700	12:00	1	Variable because of the low tempearture	700
13:00	15	80%	700	13:00	1		700
14:00	15	80%	700	14:00	1		700
15:00	15	80%	700	15:00	1		700
16:00	15	80%	700	16:00	1		700
17:00	15	80%	700	17:00	1		700
18:00	15	80%	700	18:00	1		700
19:00	15	80%	700	19:00	1		700
20:00	15	80%	700	20:00	1		700
21:00	15	80%	700	21:00	1		700
22:00	15	80%	700	22:00	1		700
23:00	15	80%	700	23:00	1		700
00:00	15	80%	700	00:00	1		700
01:00	15	80%	600	01:00	1		600
02:00	13	80%	400	02:00	1		400
03:00	11	80%	200	03:00	1		200
04:00	9	80%	100	04:00	1		100
05:00	8	80%	50	05:00	1		50
06:00	8	80%	50	06:00	1		50
07:00	8	80%	100	07:00	1		100
08:00	9	80%	200	08:00	1		200
09:00	11	80%	400	09:00	1		400
10:00	13	80%	600	10:00	1		600
11:00	15	80%	700	11:00	1		700

6.2.4 2011 *Vaccinium* cold stress time course results.

The time course, sample collecting and processing were carried out as described in 6.2.3 but the results showed little or no *CBF/DREB1* response to the cold treatments. This was possibly due to the weather conditions prevailing in Abisko whilst the samples were being collected. For example, over the week the samples were collected the weather had begun to change and night temperatures had begun to drop. It is very likely that the *Vaccinium* samples had already cold acclimated before transportation to the UK so a cold treatment of 1^{o}C would not induce a *CBF/DREB1* response once these plants were already acclimated.

6.2.5 2012 *Vaccinium* cold stress time course method

To avoid the same problems as in 2011 the time course samples were collected at midsummer in 2012 (June to early July) so that the plants were collected during the period of 24h light when the daily temperature amplitude is at a minimum, i.e. a time when the plants are least likely to be already cold acclimated.

In early July 2012 more *Vaccinium* was collected from the same location in Abisko and set up in a cold time course in the same way as described for 2011. On this occasion, a light intensity of 650µmol photons m^{-2} s^{-1} PAR (400-700nm) and ambient temperature of 16^{o}C was used, (whilst in Abisko the temperatures rose from 5^{o}C to the highest temperature of the year 23^{o}C, 16^{o}C was chosen as it is a mid-point so as not to stress the plants in ambient conditions). The light regime did not fluctuate so strongly on this occasion to match the light conditions in Abisko at that time of year, however, there would be some fluctuations in light intensity so 650µmol photons m^{-2} s^{-1} PAR (400-700nm) light intensity was chosen.

The plants were transferred to growth chambers, and samples were taken from both cold treated and ambient plants at 0, 30mins, 1 h, 2h, 3 h, 4.5h, 6h, 12 h, 24h, 48h and 72h. Samples were harvested and stored in the same manner as previously described in 6.2.4.

6.2.6 2012 *Vaccinium* cold stress time course results

qPCR experiments using cDNA made from the 2012 time course RNA did not work well. The quality of the RNA was checked with the Bio-analyser which showed that the RNA had degraded so could not be used for further qPCR experiments.

6.2.7 2013 *Vaccinium* cold stress time course, Umeå, Sweden method

After the problems with transporting plants it was decided that it would be a safer option to carry out a cold treatment time course and RNA extraction in the same place, meaning that the extracted RNA could be shipped back to the UK rather than trying to transport live plants. The RNA extractions were carried out at Umeå Plant Sciences Centre (UPSC) part of Umeå University, Sweden and the cold treatments in Skoogis, part of Sveriges lantbruksuniversitet (SLU).

Vaccinium plants from each of the three species were collected from Vindelns Försöksparker Svartloersets Fältstation, latitude 64^{o}10’ N elevation, 19^{o} 71’ E, 160-320 metres asl 60 km west of Umeå in the Västerbotten province. Large monoliths of soil and *Vaccinium* were dug out and placed into plastic trays lined with bin bags. As far as possible there were two trays per species; however, as quite often more than one species is found growing in each area there were some mixed monoliths. The plants were then transported to Skoogis, Sveriges lantbruksuniversitet (SLU) in Umeå to two pre-set environmental rooms (Karl Weiss, Giessen, Germany). All of the plants were put into the ambient room set to 17.5^{o}C, 70% humidity and 230 µmol photons m^{-2} s^{-1} light with 2 hours of reduced light to almost darkness to match the conditions outside. After a period of three days to grow accustomed to the conditions, half of the plants were moved to an identical environmental

room with matching light but a temperature of 2°C. Time course samples were taken in duplicate for all three species in both cold and ambient rooms at time points 0h, 1h, 2h, 3h, 4.5h, 6h, 12h, 24h, 48h, 72h. All samples were flash frozen in scintillation tubes in liquid nitrogen and stored in at -80°C. RNA was then extracted from each sample at Umeå University Plant Sciences Center (UPSC) following the method described in section 2.8.1.1. There was one important change from the method previously used in that a tissuelyser was used to break up the tissue by shaking at high speed with a ball bearing inside (whilst still frozen). Each sample was split into three eppendorfs due to the volume of ground tissue and buffer and then combined after the chloroform clean up stage when the volume of extract was around 500µl (before adding lithium chloride). All of the extracted RNA was measured using a nanodrop (See 2.8.4.1) and stored at -80°C. The samples were then shipped on dry ice back to the UK for subsequent DNase treatment (see 2.9.1.1), cDNA synthesis and qPCR (see 2.12.1.1)

6.2.8 2013 *Vaccinium* time course Umeå, Sweden results.

All of the following qPCR results throughout Chapter 6 were carried out using the samples collected in the 2013 time course experiment. The sequencing reactions detailed were carried out using RNA extracted from the preliminary 2010 cold treatment samples.

6.2.9 *DREB1/CBF* expression in response to cold

The first experiments were designed to measure the expression of *CBF/DREB1* after the extended time course (Figure 6-2).

From the results it would appear that *V. myrtillus* has a peak in *CBF/DREB1* expression after 2h with a second peak after 24h exposure to cold. However the initial peak in expression at the 0 time point and the increase at 4.5h ambient are anomalous. The *V. uliginosum* expression levels were consistently high in the ambient samples rather than the cold suggesting that the plants may have higher *CBF/DREB1* expression at 16°C. At 48h, expression in the cold treated sample is higher than the ambient sample but as the ambient samples are high it is difficult to determine whether this increase in expression is due to the cold treatment or another factor. As the ambient samples have high expression it is possible that the cold samples have an increase in expression too but just not to the same extent as the ambient samples. The difference in expression level is due to the difference in temperature as all other variables were the same between rooms except the actual plant itself as multiple plants were used.

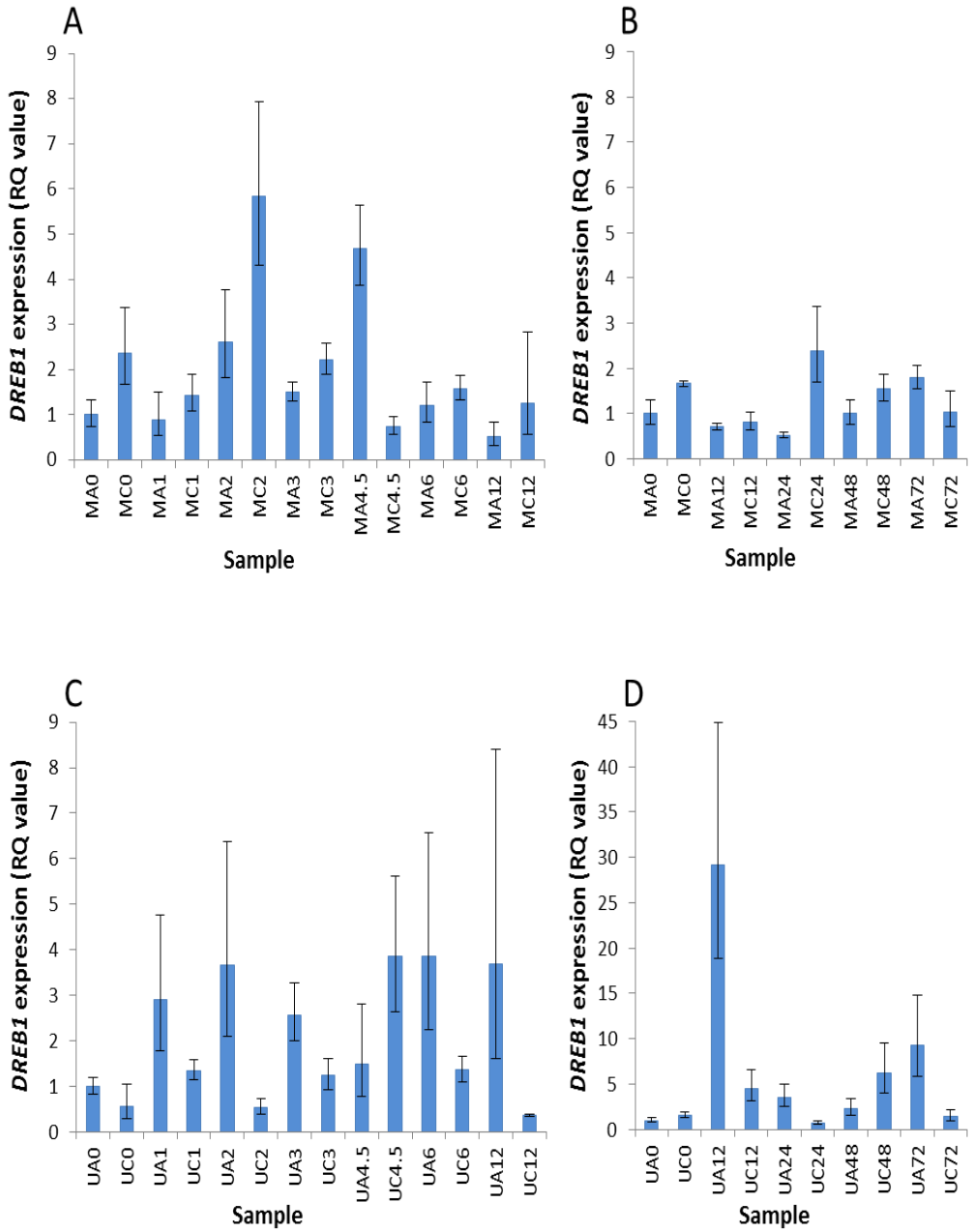
The *V. vitis-idaea* results are the most clear as there is a high peak in expression after 1h of exposure to cold and a small increase after 3h exposure to cold, the rest of the cold treated and ambient time points are uniformly low.

To reduce the technical error for each sample in an attempt to make the results clearer the qPCR for time points 0h to 3h were repeated with each sample having five technical replicates rather than the usual method of triplicate technical replicates. These time points were chosen based on the samples which showed the most variability in *CBF/DREB1* expression and the fact that *Arabidopsis* has a peak in *CBF/DREB1* expression after 2h.

From Figure 6-3 the *V. myrtillus* results still show an increase in *CBF/DREB1* expression after 2h exposure to cold. However the both the 3h cold and ambient samples show an increase in expression and to almost the same level possibly suggesting a circadian increase in *CBF/DREB1* (Fowler et al., 2005).

V. uliginosum does not display a clear increase in *CBF/DREB1* expression for any of the time points when using 5 technical replicates. UC1 is higher than the ambient at 1h but it is not statistically higher. The 3h time points being elevated matched the *V. myrtillus* 3h time points again suggesting a circadian increase in expression.

V. vitis-idaea again in Figure 6-3 has a large increase in expression after 1h in the cold and again increases slightly after 3h exposure to cold when using five technical replicates.


A
DREB1 expression (RQ value)
0
1
2
3
4
5
6
7
8
9
MA0
MC0
MA1
MC1
MA2
MC2
MA3
MC3
MA4.5
MC4.5
MA6
MC6
MA12
MC12
Sample
B
DREB1 expression (RQ value)
0
1
2
3
4
5
6
7
8
9
MA0
MC0
MA12
MC12
MA24
MC24
MA48
MC48
MA72
MC72
Sample
C
DREB1 expression (RQ value)
0
1
2
3
4
5
6
7
8
9
UA0
UC0
UA1
UC1
UA2
UC2
UA3
UC3
UA4.5
UC4.5
UA6
UC6
UA12
UC12
Sample
D
DREB1 expression (RQ value)
0
5
10
15
20
25
30
35
40
45
UA0
UC0
UA12
UC12
UA24
UC24
UA48
UC48
UA72
UC72
Sample

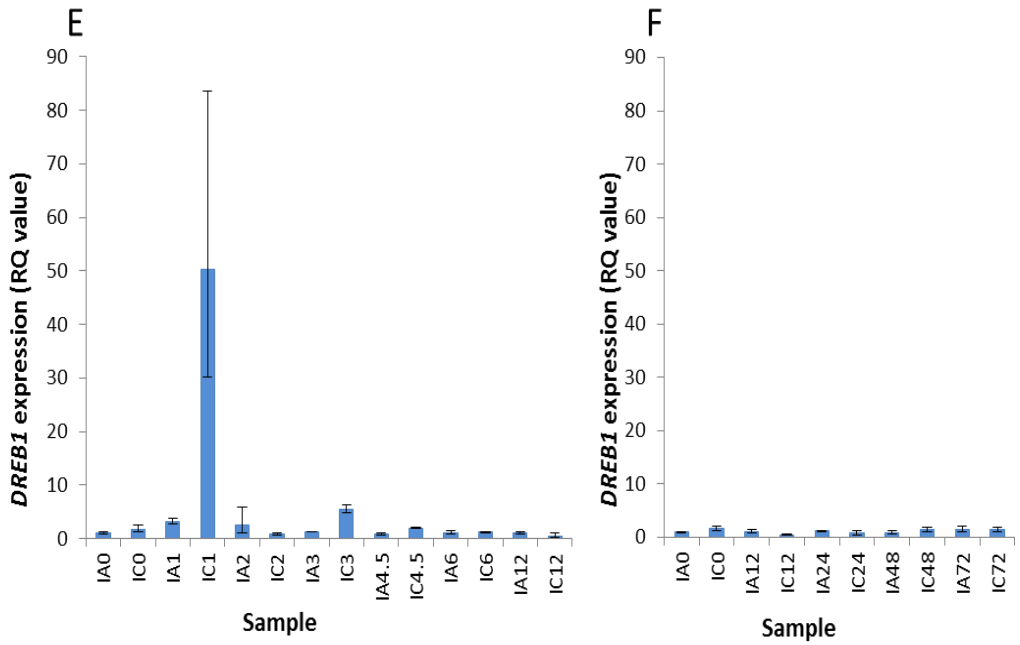


Figure 6-2: qPCR results for *CBF/DREB1* using an extended time course of time points 0, 1,2, 3, 4.5, 6, 12, 24, 48 and 72 hours for samples after both cold (c) temperatures of 2^{o}C and ambient (a) temperatures of 17.5^{o}C. From each of the three *Vaccinium* species, *V. myrtillus* (M) graphs A and B, *V. uliginosum* (U) graphs C and D, *V. vitis-idaea* (I) graphs D and E, samples are normalised to the 0h Ambient control for their respective species. Error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Student's *t*-test.

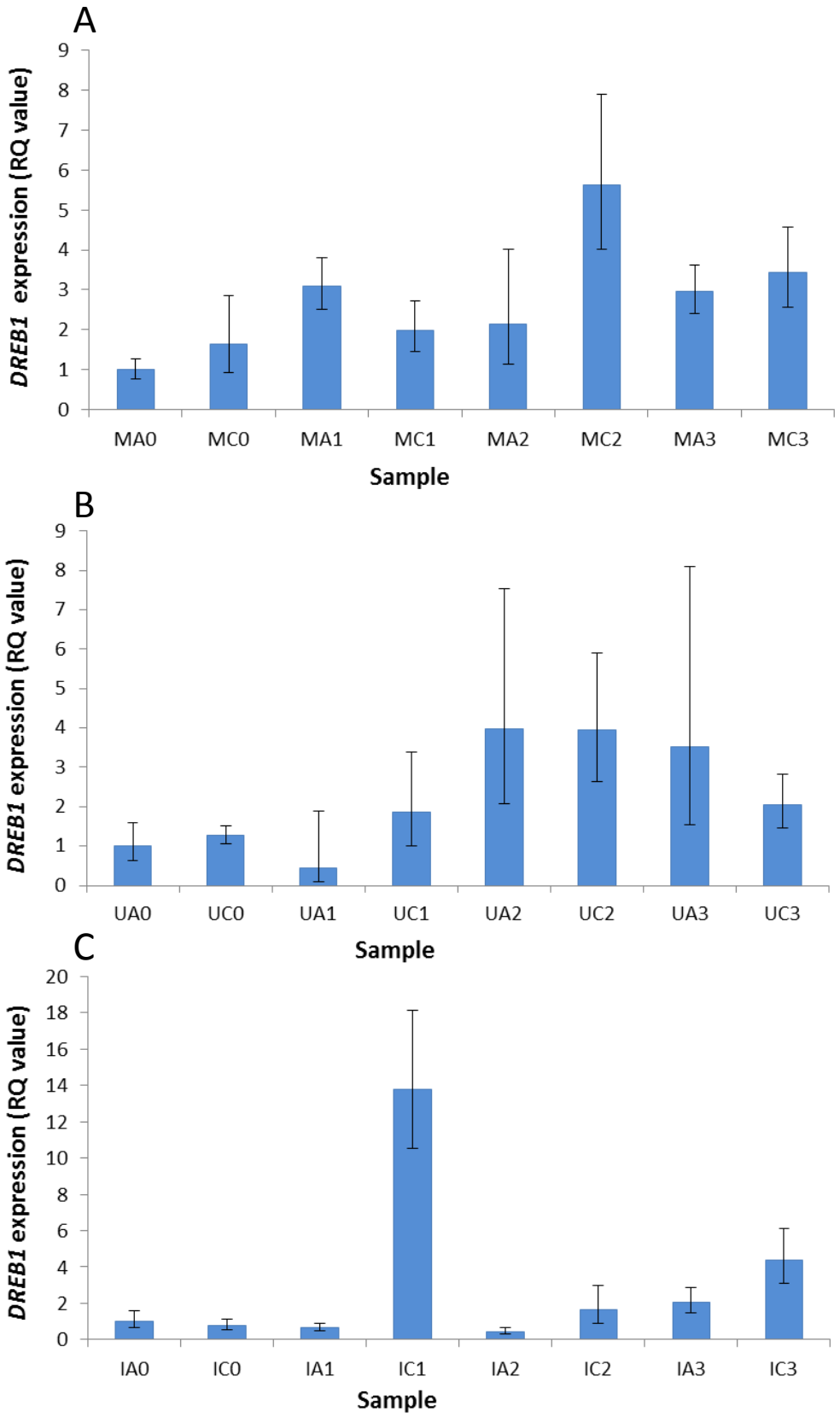


Figure 6-3: qPCR results for *CBF/DREB1* expression with 5 replicate wells for each sample. Time points 0, 1, 2, 3h for samples after both cold (c) temperatures of 2°C and ambient (a) temperatures of 17.5°C. From *V. myrtillus* (M), *V. uliginosum* (U) and *V. vitis-idaea* (I). Samples are normalised to the 0h ambient control for their respective species. Error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Students *t*-test.

6.3 Expression levels of other components of the CBF/DREB1 dependent and independent pathways.

To try to understand the difference in expression levels between the three *Vaccinium* species in response to cold, other proteins and transcription factors controlling parts of the CBF/DREB1 pathway were identified along with some possible CBF/DREB1 independent genes. A pathway involving these factors was constructed to identify easily their function (Figure 6-4). Upstream proteins of the CBF/DREB1 pathway were highlighted as they could further confirm the results from the *CBF/DREB1* time course expression. Alternatively the other proteins and transcription factors could help to explain results by showing what is happening upstream of CBF/DREB1 in *Vaccinium* to either cause or inhibit changes in expression of *CBF/DREB1*, such as in *V. uliginosum* which doesn't appear to have much of a *CBF/DREB1* response to cold.

Two CBF/DREB1 independent expression pathways were chosen as possible alternative routes for *Vaccinium* to use in response to cold. In particular the eskimo pathway as it has been associated with high accumulation levels of proline (Xin & Browse, 1998) and *V. uliginosum* has been reported to contain high levels of proline (Stewart & Bannister, 1973).

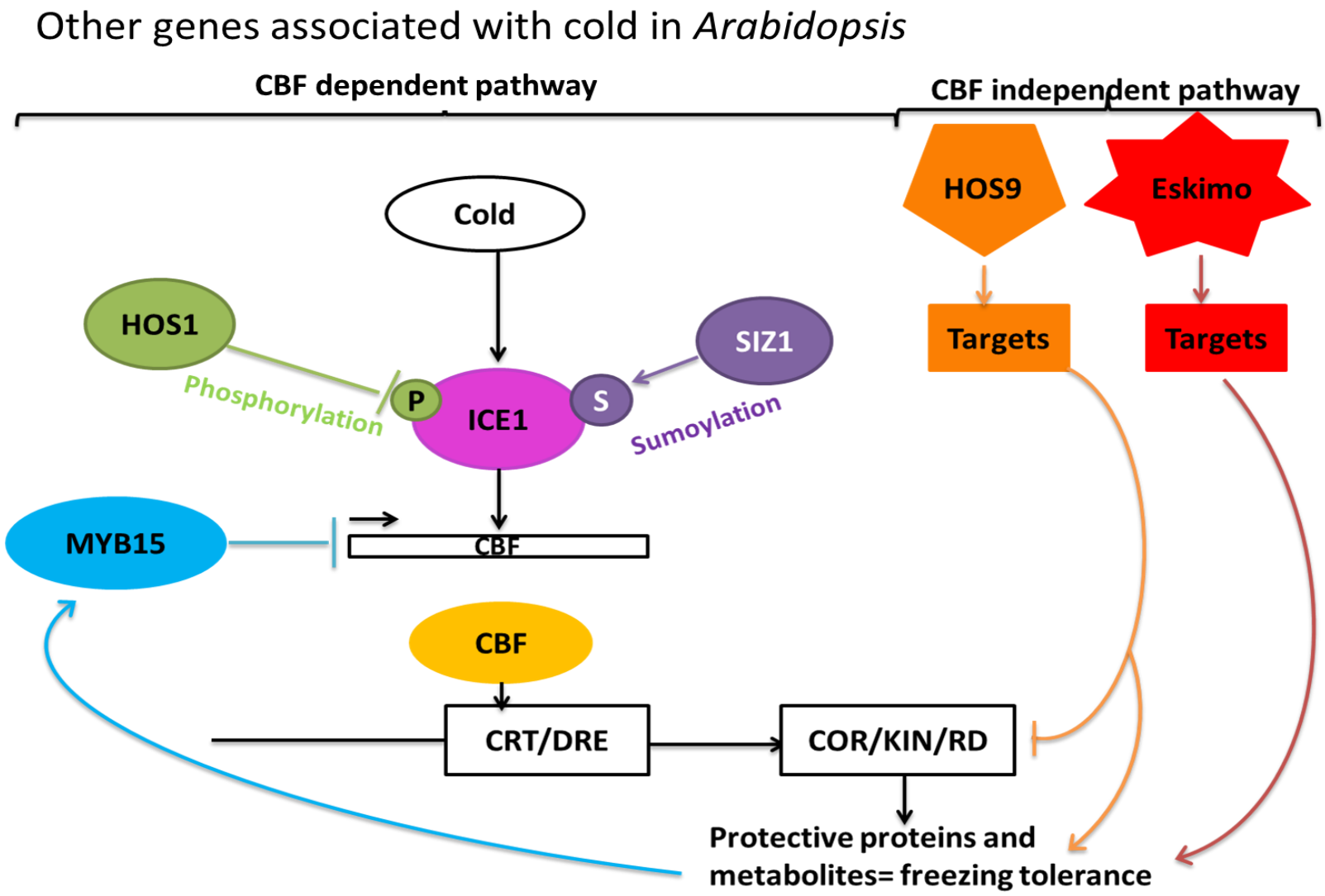
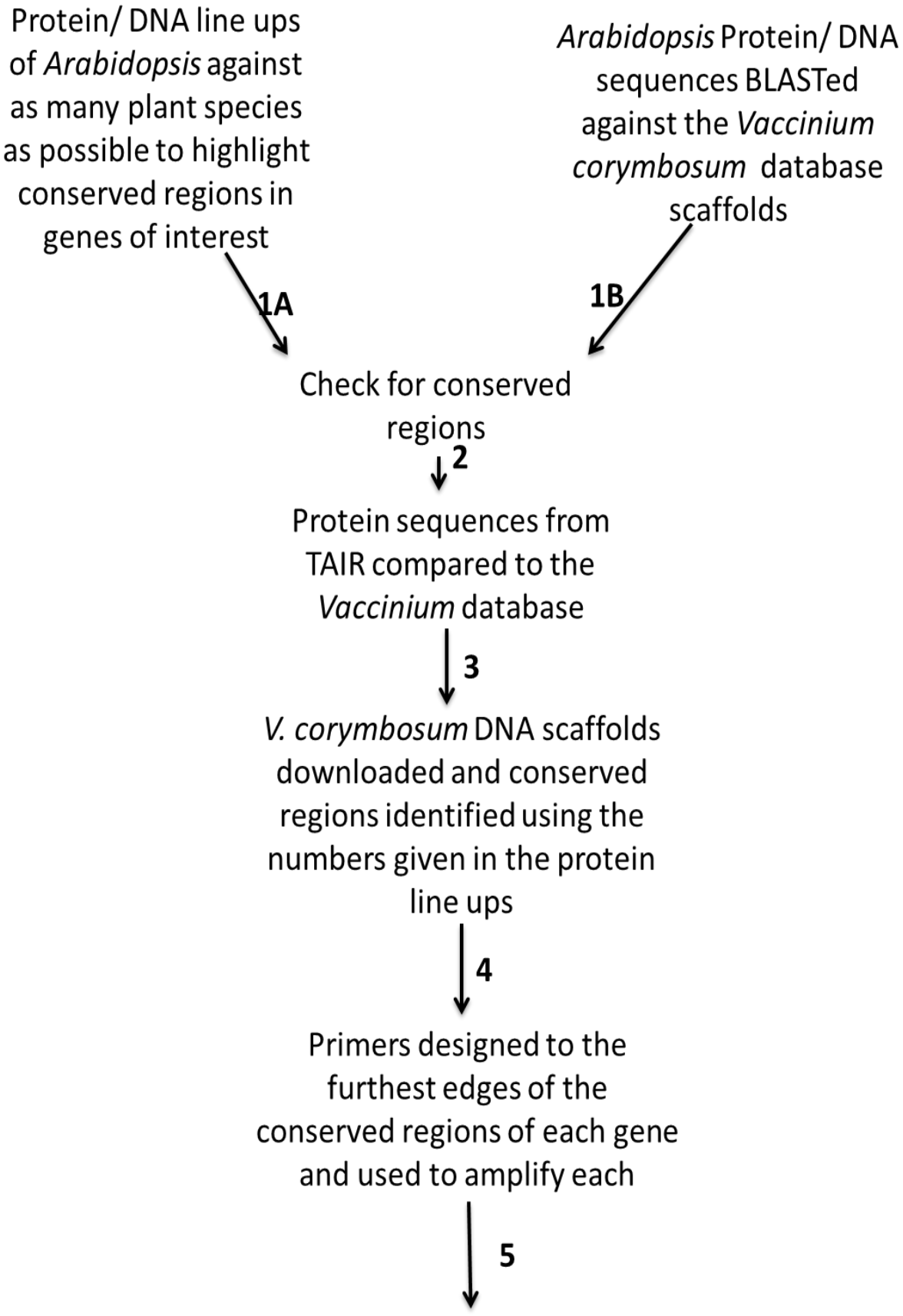


Figure6-4: Other proteins associated with the DREB1/CBF pathway and DREB1/CBF independent pathways


Protein/ DNA line ups of *Arabidopsis* against as many plant species as possible to highlight conserved regions in genes of interest
Arabidopsis Protein/ DNA sequences BLASTed against the *Vaccinium corymbosum* database scaffolds
1A
1B
Check for conserved regions
2
Protein sequences from TAIR compared to the *Vaccinium* database
3
V. corymbosum DNA scaffolds downloaded and conserved regions identified using the numbers given in the protein line ups
4
Primers designed to the furthest edges of the conserved regions of each gene and used to amplify each
5

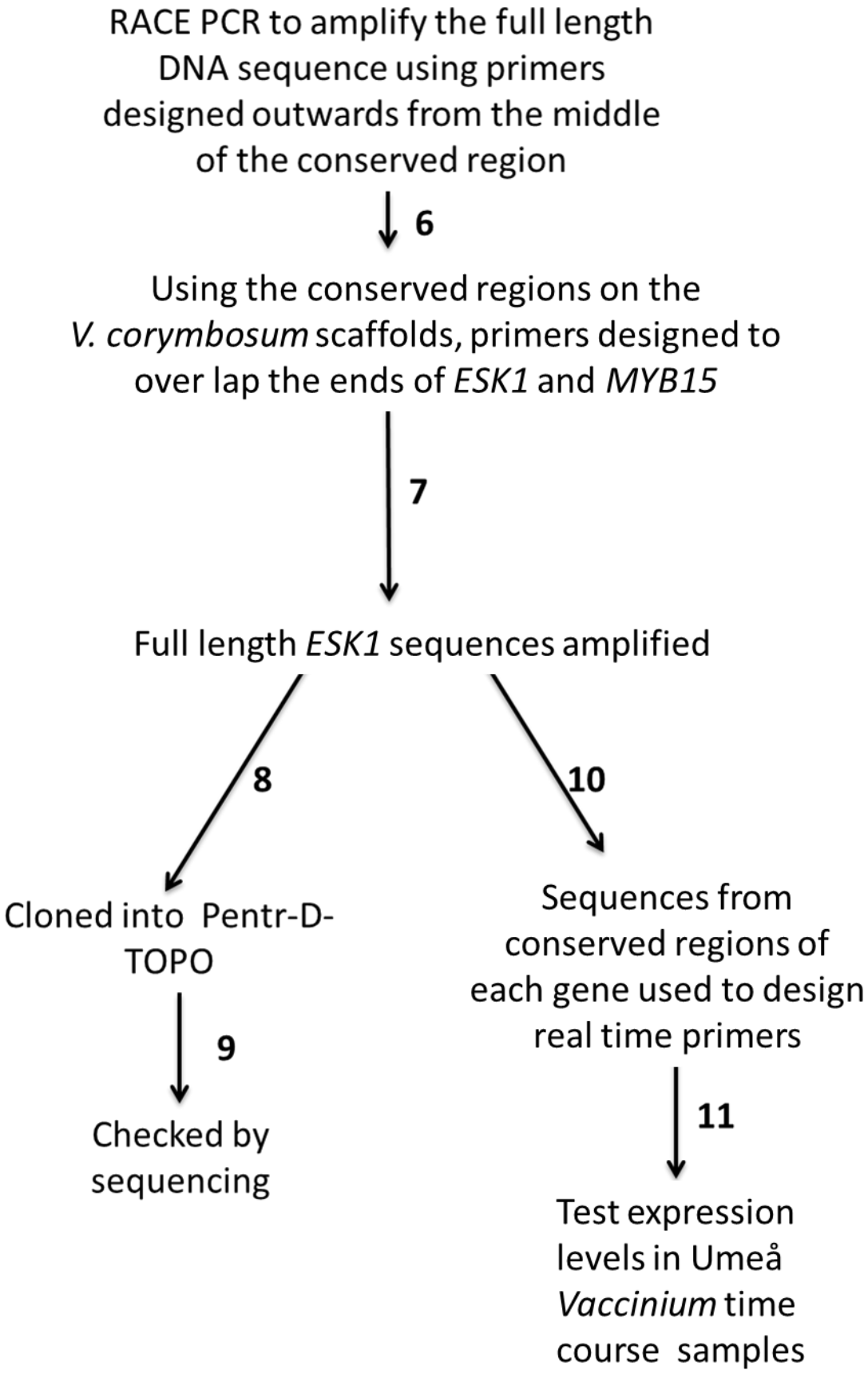


Figure 6-5: A flow chart to show the scheme of work and methods used to obtain the partial *Vaccinium* non *CBF/DREB1* sequences and the real time primers used later in the chapter.

6.3.1 Identification and sequencing of non-*CBF/DREB1* genes

When this project started in 2010 no genome sequence for any *Vaccinium* species had been sequenced. Since then a project to sequence the genome of *Vaccinium corymbosum* (American blueberry) has started, however, it has not been completed. Primary scaffolds and contigs are available to BLAST search against through a *Vaccinium* database website. However specific genes and annotations of any kind currently remain unavailable (Rowland et al., 2012).

Measuring expression levels of other genes involved in the CBF/*DREB1* pathway and the non *CBF/DREB1* pathway is therefore much more difficult than in sequenced species such as *Arabidopsis.*

Following the flow chart in Figure 6-5 two different starting points were attempted simultaneously (Figure 6-5 A and B), the first was to examine both DNA and protein line ups for as many plant species as possible (Appendix E –Clustal/Jalview line ups of *ESK1*) in comparison to the chosen gene in *Arabidopsis*. This was to identify any areas that were highly conserved between the different species. As there is a lot of variation between the different species and some genes sequences were only available from relatively few species a different approach was used in addition. The primary scaffolds for the *V. corymbosum* genome sequence are available on NCBI (www.ncbi.nlm.nih.gov) and can be used in BLAST searches on the *V. corymbosum* genome website (www.*Vaccinium*.org). Using both DNA and protein sequences from TAIR (www.arabidopsis.org) it was possible to BLAST (Altschul et al., 1997) search for areas of the primary scaffolds that matched the genes of interest. This then gave fragments of DNA sequence that could be checked against the DNA line ups from Figure 6-5 1A and B. However, as there is a lot of sequence variation it was difficult to establish whether the right gene had been identified. This method was tried again using protein sequence and tblastn against the *V. corymbosum* scaffolds. This gave line ups of protein sequence with numbers at the end that correspond to the exact DNA bases that code this region. The relevant scaffolds were then downloaded from NCBI and regions of interest highlighted. Primers were designed to the furthest edges of each gene, which were then used to amplify and sequence the same fragments from the three species of Arctic *Vaccinium.*

For sequences such as *SIZ1* it was difficult to isolate just one *SIZ1* sequence as in *Arabidopsis* there are five *SIZ1* genes that are all similar in sequence. When sequencing the fragments of *Vaccinium SIZ1* there are also multiple copies of *SIZ1* but without the individual sequences of each *SIZ1* gene it is impossible to design primers that are specific to just one copy of the gene. The sequencing results showed multiple copies of the *SIZ1* gene

which made the sequence results impossible to interpret because the chromatograms comprised of multiple sequences intertwined so it was not possible to determine which peaks corresponded to the relevant version of *SIZ1,* figure 6-6.

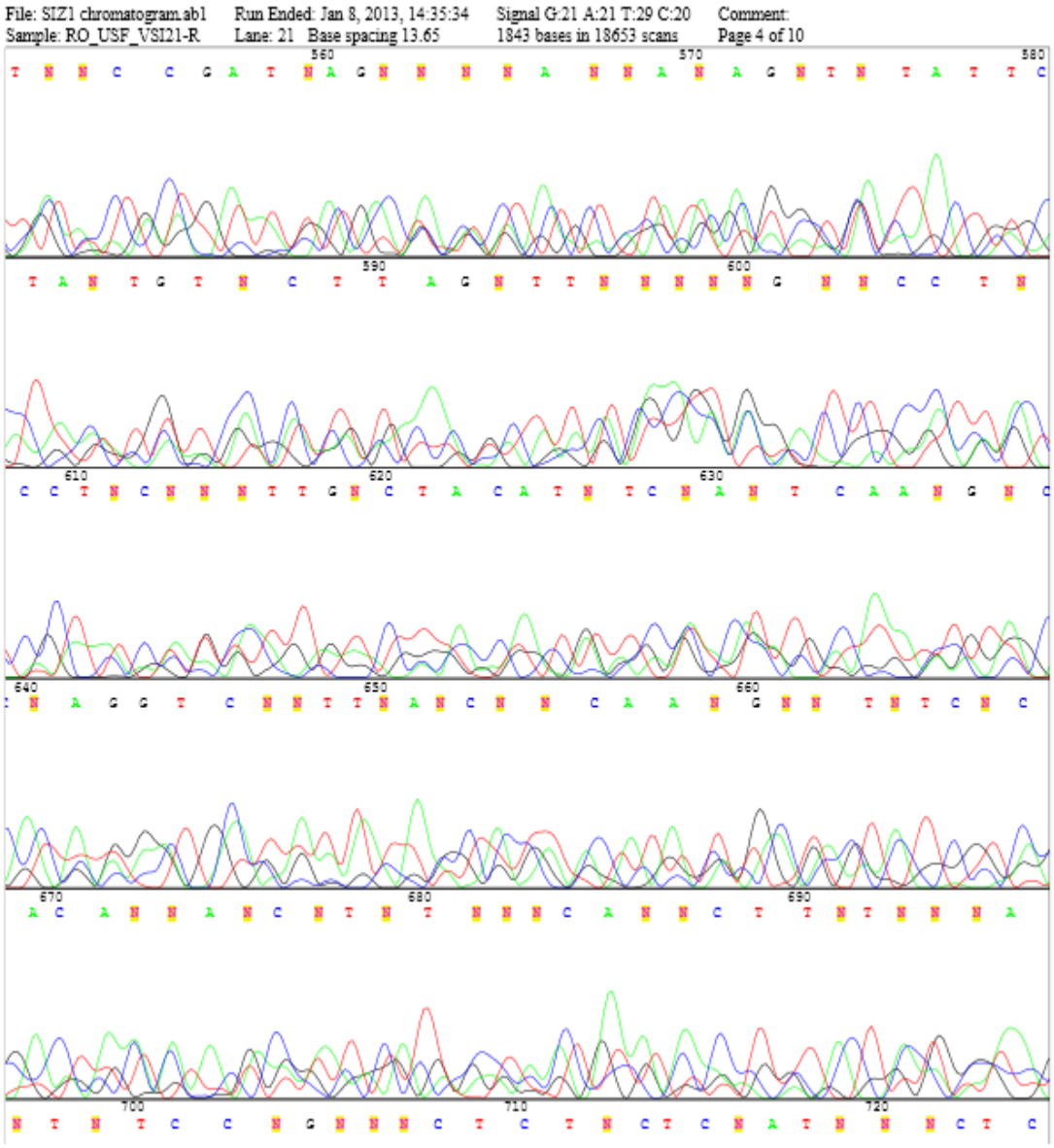


Figure 6-6: Sequencing chromatograms for SIZ1 from *V. uliginosum,* displayed using Chromas lite 2.0 (Technelysium pty Ltd, Brisbane, Australia).

To identify the 5' and 3' ends of the coding region for each gene, and to sequence the full length, RACE PCR was used with three nested primers to carry out both 3' and 5' RACE (see 2.10.3). The RACE PCR was unsuccessful, possibly due to the quality of the RNA used, and it was not possible to tell the specificity of the primers so it is likely that they hybridised to more than one gene at a time causing the reaction to fail.

As the RACE PCR was un-successful a different approach was attempted. Going back to the *V. corymbosum* genome database and the previously identified relevant scaffolds it was possible to identify the beginning and end of each gene from the sequences that were already highlighted by working back to look for any stop and start codons close to the highlighted regions within the scaffold using *Arabidopsis* as a template. However, because of the level of variation between the species and the presence of multiple introns in genes it was not possible to predict accurately the length of the genes including introns, and therefore to identify the ends reliably. For *ESK1* and *MYB15* both the start and stop codons were identified and primers were designed to each end with the aim of amplifying the full length of the gene. *MYB15* did not amplify for any of the three *Vaccinium* species and it was not considered useful to continue given the time frame. *ESK1* did amplify and was cloned into pENTR-D TOPO (see 2.13) to sequence and ultimately recombine into a vector to transform *Arabidopsi*s for overexpression studies. Each time the amplified *ESK1* was cloned it was sequenced to check that the right gene had been amplified. Multiple genes were cloned successfully but unfortunately none of them was *ESK1*; there are many genes with similar beginning and end regions to *ESK1* in *Arabidopsis* and it appears this may be the same in *Vaccinium*. This meant that the original plan of cloning the full length Eskimo gene to transform an *ESK1* mutant line in *Arabidopsis* had to be abandoned as it would not have been possible to produce stable transformants in the given time frame. Instead, the short fragments of each gene identified in Figure 6-5 part 5 were re-amplified and cloned (see 2.13) to allow each fragment to be sequenced clearly, with each clone only containing one sequence. Although this could not provide the full length sequences of each gene the short fragments were large enough to be used to design real time primers which were then used to test the expression levels of these genes in the extended time course experiment.

6.3.2 Partial *Vaccinium* non *CBF/DREB1* sequences

The partial sequences from all three Arctic *Vaccinium* species were aligned using Clustal Omega (Sievers et al., 2011) to allow comparisons to be made between the three species and in most cases it allowed the same primer set to be designed for all three species (Appendix C –List of primers). Line ups were made using Clustal omega and then jalview

(Waterhouse et al., 2009) to display differences between sequences more clearly. (Figure 6-7 to 12)

6.3.3 Checking the partial sequences of the non-*CBF/DREB1* genes

Two different methods were used to check the partial sequences were the right genes. The first was carried out using BLAST2 (Tatusova & Madden, 1999) to compare the partial sequences obtained to the full genomic sequence for each gene in *Arabidopsis* (Table 6.7).

Table 6.7: BLAST2 line ups of partial *Vaccinium* sequences against genomic *Arabidopsis* sequences for each gene. Table shows the percentage of gene cover for each sequence compared to the full length and the percentage of positively matched bases within each sequence.

	V. myrtillus		*V. uliginosum*		*V. vitis-idaea*	
	cover	positives	cover	positives	cover	positives
MYB15	11	93	34	93	37	93
HOS1	19	100	18	100	17	100
HOS9	48	100	44	89	21	89
ICE2	61	75	57	73	44	76
SIZ1	71	67	57	67	63	67
ESK1	51	49	49	80	28	76

The percentage of some genes that were positively matched is quite low and the amount of cover of some genes is also quite low. It is possible that the wrong gene was isolated as there may be regions of conservation between two completely different genes. This was also shown from the results in Table 6.7 where it was possible to clone a number of different genes when trying to clone the full length *ESK1* gene.

To avoid this problem, the partial *Vaccinium* sequences were aligned to the gene they match most closely in *Arabidopsis* using TAIR BLAST (www.arabidopsis.org) (Table 6.8).

Table 6.8: Results of TAIR BLASTtn NT to AA searches of partial *Vaccinium* sequences against their nearest match gene in *Arabidopsis*.

	V. myrtillus	*V. uliginosum*	*V. vitis-idaea*
MYB15	MYB15	MYB15	MYB15
HOS1	MEE62	MEE62	MEE62
HOS9	SOUL	AT3G01380	no match
ICE2	ICE2	ICE2	ICE2
SIZ1	SIZ1	SIZ1	SIZ1
ESK1	ESK1	ESK1	AT1G09400

The combined results of TAIR BLASTtn and BLAST2 showed that HOS1 and HOS9 were not reliably the right genes. In particular the sequences for *HOS1* had low levels of coverage and when checked against the *Arabidopsis* genome had the closest match to *MEE62* in all three *Vaccinium* species. For *HOS9,* although the *Vaccinium* sequences had a reasonable amount of sequence coverage the TAIR BLASTtn results found their closest match to be different genes and in contrast to *HOS1* each species had a different gene they matched to. For this reason it was decided that *HOS1* and *HOS9* should not be used for expression studies in the time courses as it was not certain the expression of which genes was actually being measured.

The *Vaccinium ICE1* sequences were found to match *ICE2* more closely which is why all further references to *ICE* genes are to *ICE2* instead of *ICE1*. However, this should not have altered the overall outcome as *ICE2* has a similar function to *ICE1* in *Arabidopsis* (Fursova et al., 2009).

V. vitis-idaea ESK1 sequence was also another outlier and BLAST searches showed it to be most closely matched to a *AT1G09400* (FMN-linked oxidoreductases superfamily protein). However as both the *V. myrtillus* and *V. uliginosum* sequences matched *ESK1* and had similar sequences to *V. vitis-idaea* it was possible to design qPCR primers that could be used for all three species, it is therefore possible that *ESK1* expression level was measured for *V. vitis-idaea* too.

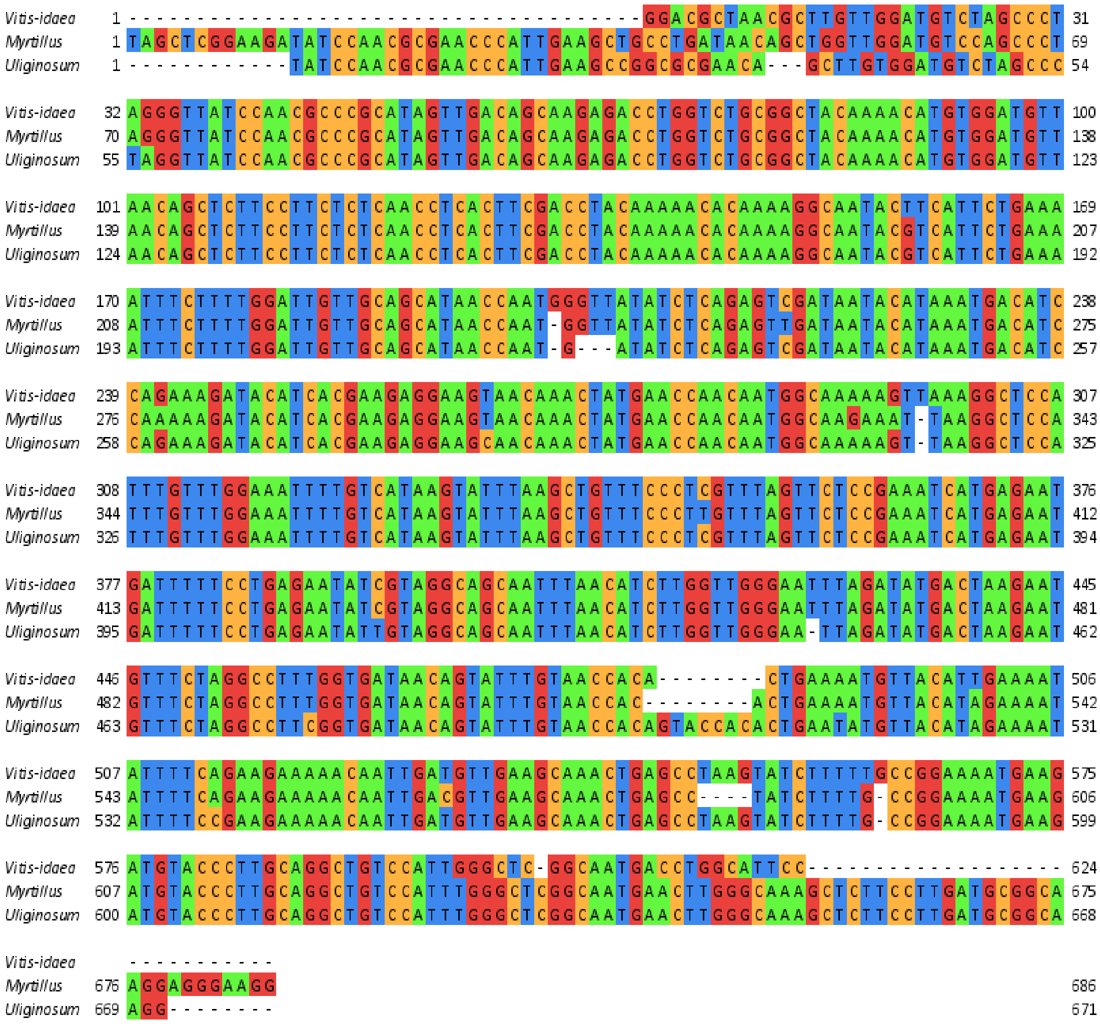


Figure 6-7: Clustal omeaga/ Jalview line ups of partial ICE2 DNA sequences from *V. myrtillus*, *V. uliginosum* and *V. vitis-idaea*. qPCR primers were designed to the same region of sequence homology for all 3 species.

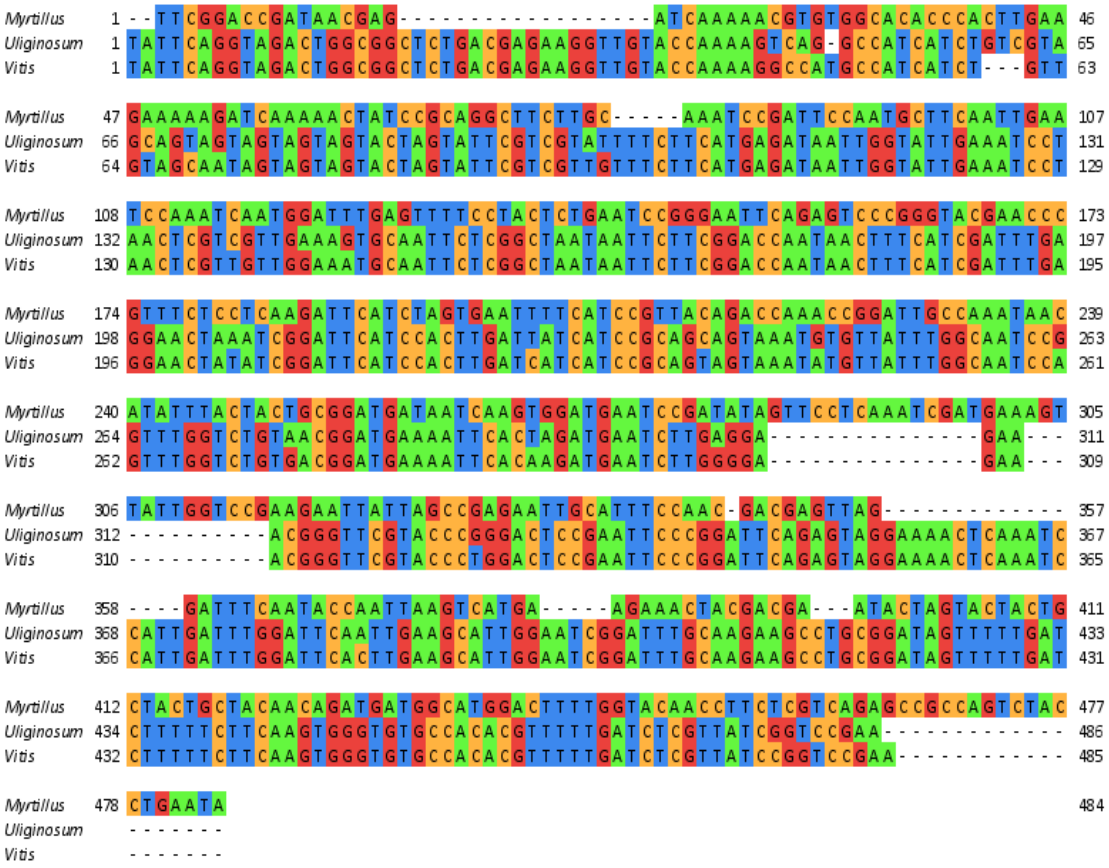


Figure 6-8: Clustal/ Jalview line ups of partial MYB15 DNA sequences for *V. myrtillus*, *V. uliginosum* and *V. vitis-idaea*. qPCR primers were designed to the same region of sequence homology for all three species.

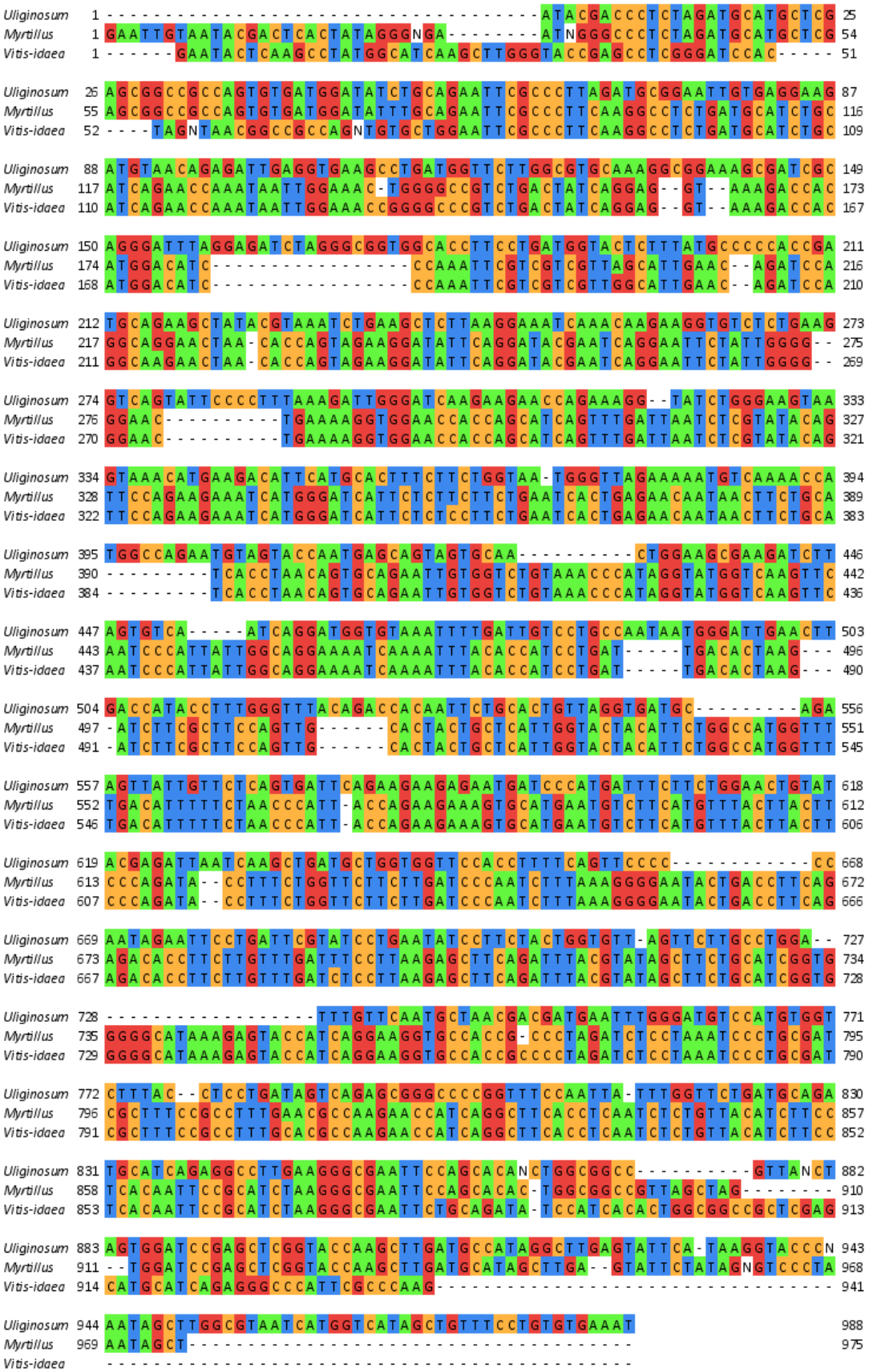


Figure 6-9: Clustal omega/ Jalview partial SIZ1 DNA sequence alignments for *V. myrtillus*, *V. uliginosum* and *V. vitis-idaea*. Different primers were designed for *V. vitis-idaea* due to the large differences in sequence compared to the other two species.

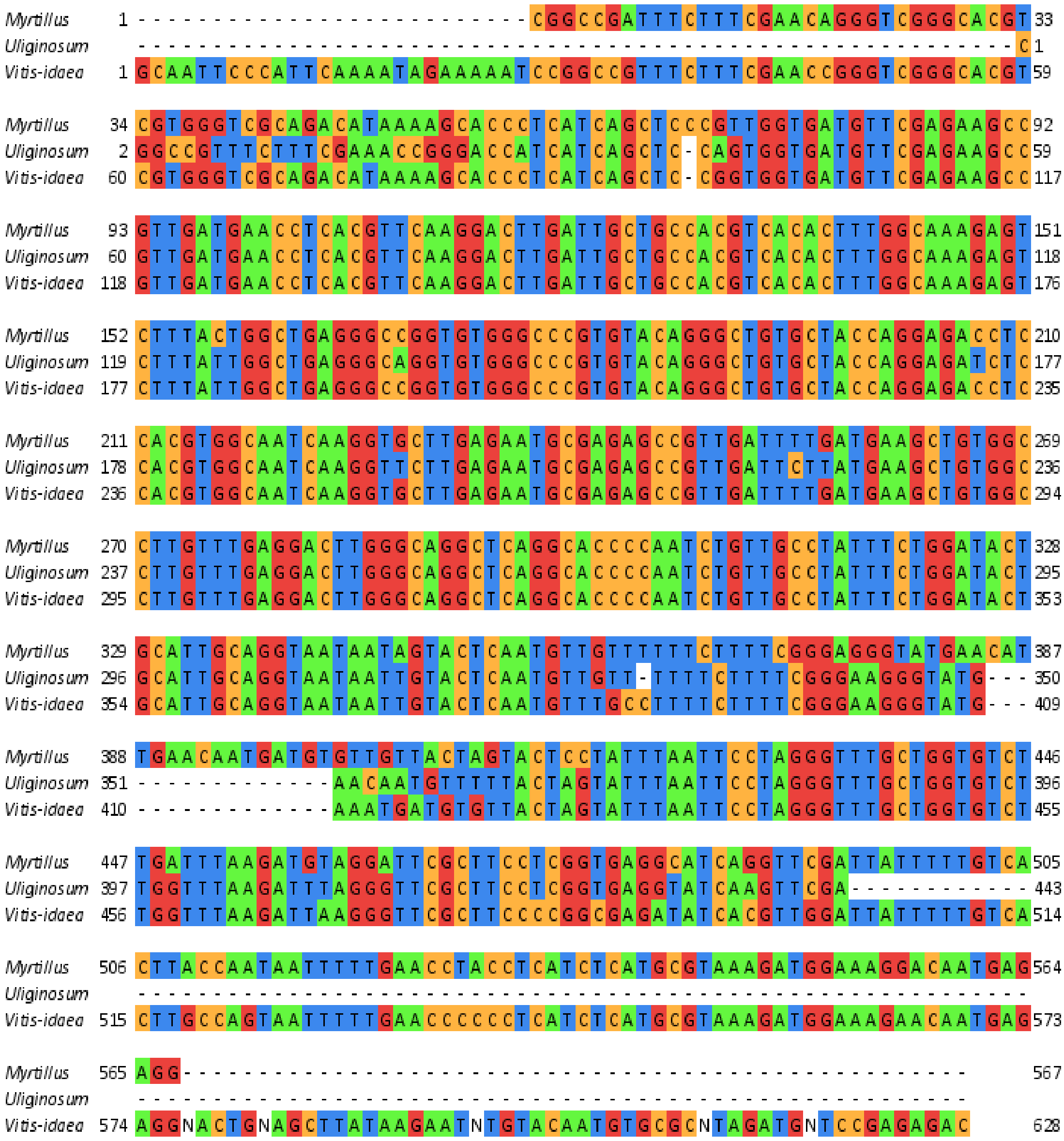


Figure 6-10: Clustal omega/ Jalview line ups of partial HOS1 DNA sequences for *V. myrtillus, V. uliginosum* and *V. vitis-idaea.*

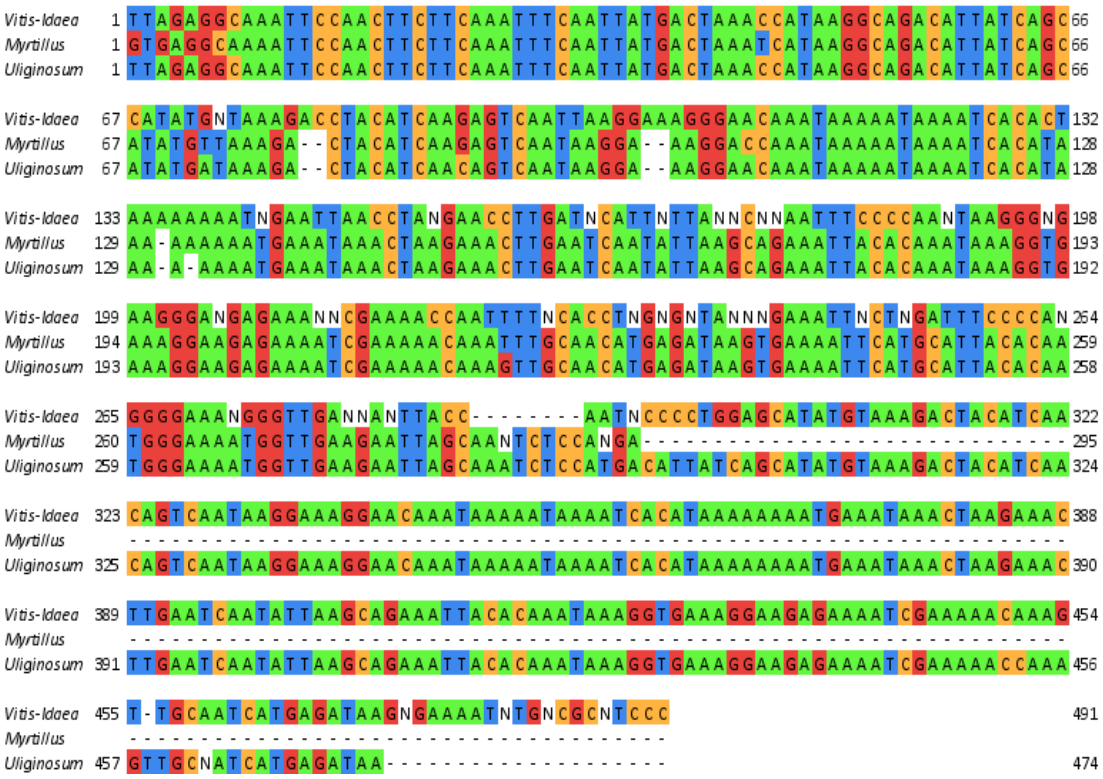


Figure 6-11: Clustal omega/ Jalview line ups of partial HOS9 DNA sequences for *V. myrtillus*, *V. uliginosum* and *V. vitis-idaea*.

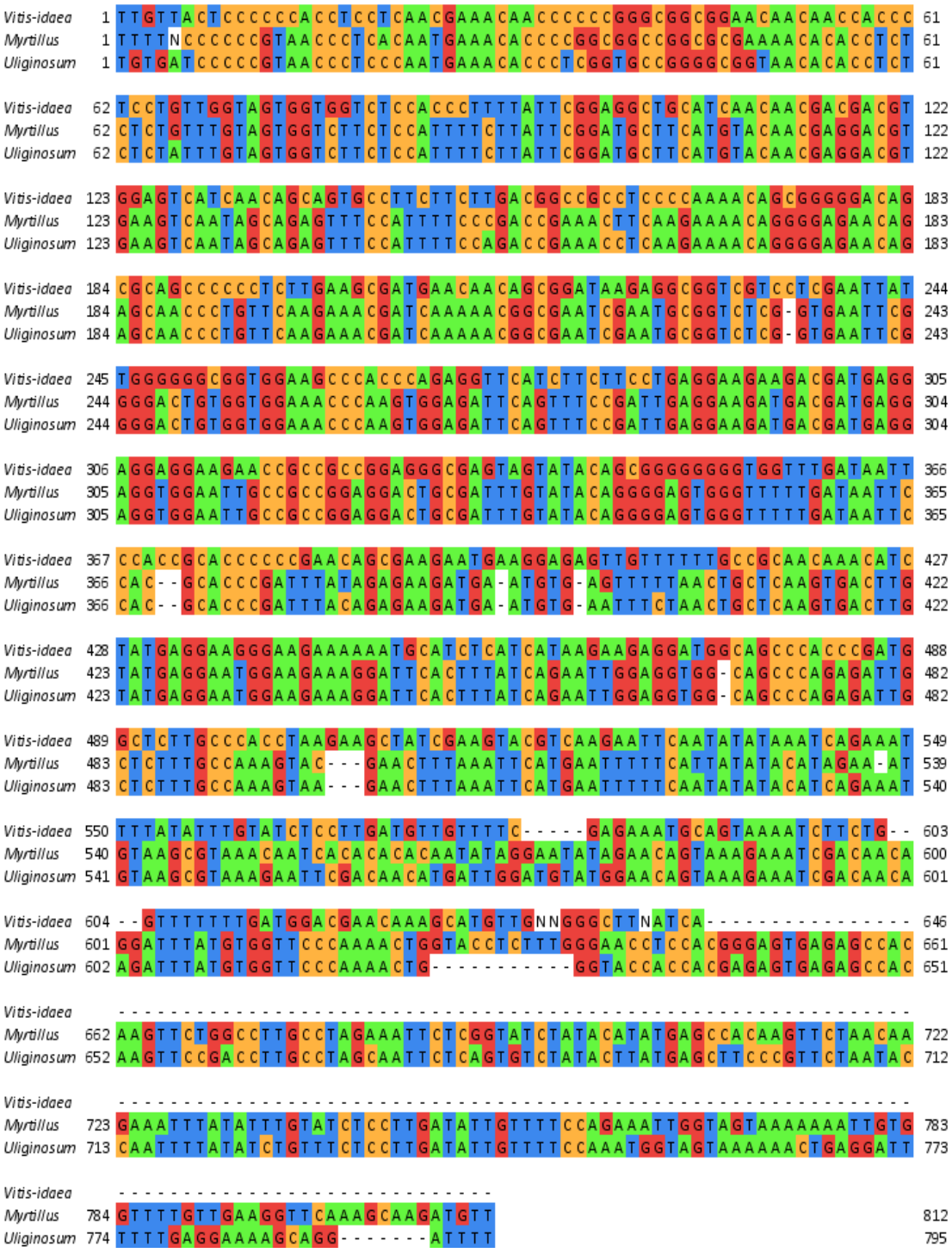


Figure 6-12: Clustal omega/ Jalview line ups of partial ESK1 DNA sequences for *V. myrtillus*, *V. uliginosum* and *V. vitis-idaea*. The same qPCR primers were used for all three species due to the sequence homology between all three species at the start of the partial sequence.

6.3.4 Expression levels of other genes involved in freezing tolerance

Using the primers designed in section 6.3.2 and the RNA extracted from the 2013 *Vaccinium* time course, qPCR expression levels were tested for *ICE2, MYB15, SIZ1* and *ESK1*.

6.3.4.1 ICE2

In *Arabidopsis* it is thought that *ICE2* is constitutively expressed across all tissues within the plant (Fursova et al., 2009).

From the qPCR results in Figure 6-13 A and B only the 1h and 6h cold samples are significantly lower than the control samples. It is difficult to say for certain what the *ICE2* expression pattern for *V. myrtillus* is as the error bars are large and mostly overlap each other. This could have been caused by problems with the *ICE2* primers which had a tendency to form primer dimers in one of the three wells for each sample causing a large amount of variation in each sample for *V. myrtillus* and therefore increasing the error.

V. uliginosum had no results in this figure for *ICE2* expression as the results were repeatedly undetectable, consistent with the low levels of *CBF/DREB1* expression.

V. vitis-idaea has clear expression of *ICE2* from Figure 6-13 C sample (IC1) after 1h in the cold whereas all of the other samples have low expression by comparison.

The large error bars for *V. myrtillus* in Figure 6-14 make the five well repeat qPCR more important as increasing the number of repeats should reduce the amount of uncertainty in each sample. From Figure 6-14 the *V. myrtillus* samples look as though there may be an increase in *ICE2* expression after 3h in response to cold, however this result is not significant due to the error bar overlap with the control samples. The most noticeable difference for *V. myrtillus* is the significant difference in *ICE2* expression between the cold and ambient 0h controls. The ambient 2h sample is significantly higher than both the cold and ambient 0h controls.

V. vitis-idaea in Figure 6-14 has an increase in expression for each sample in response to cold when compared to the ambient at each time point. However, the large error bars again make it difficult to say for certain. Again the cold 0h control is significantly higher than the 0h ambient control. At 2h there is also a significant increase in the cold treated sample when compared to both the ambient 2h sample and the ambient 0h sample.

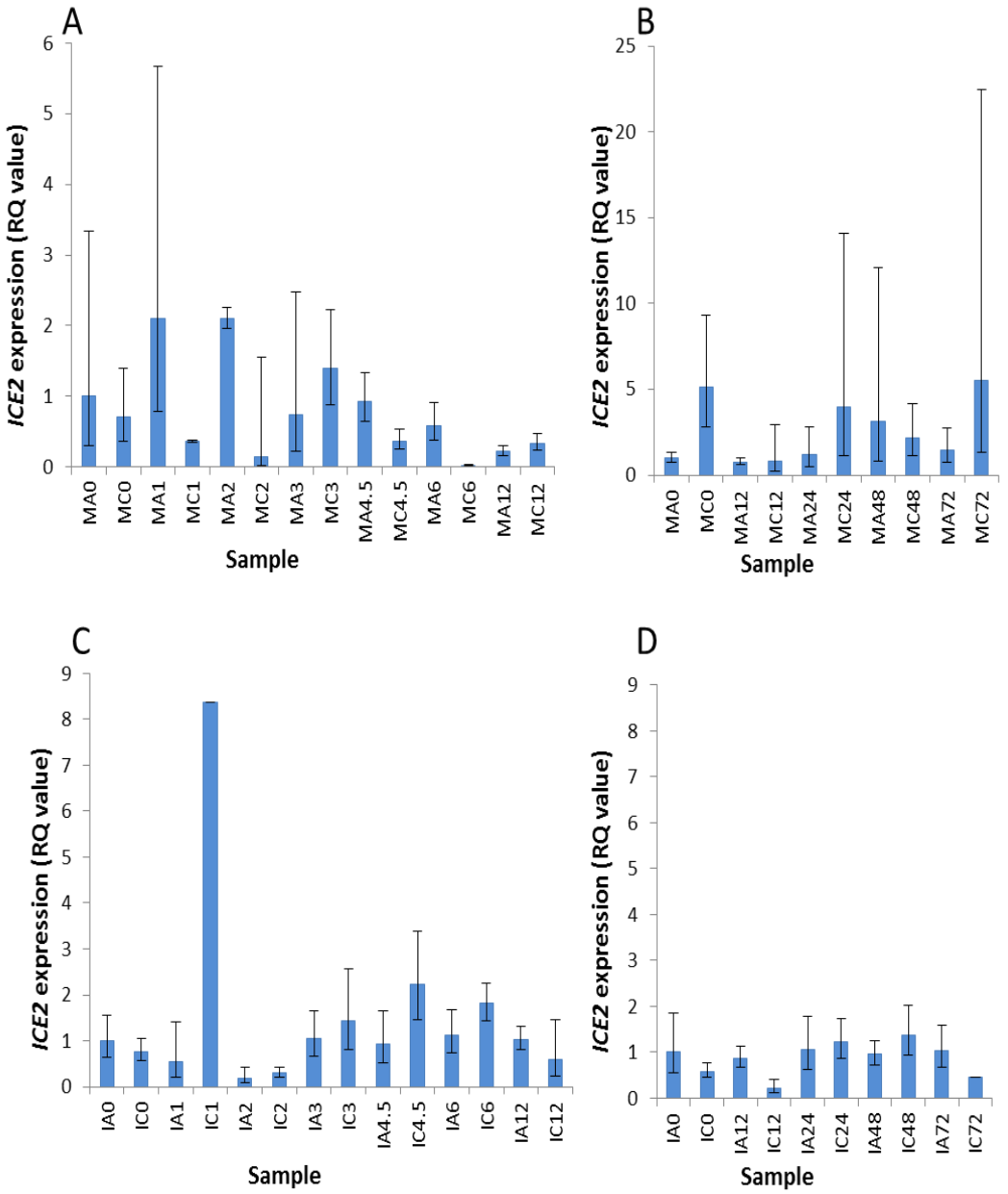


Figure 6-13: qPCR results for *ICE2* using an extended time course of time points 0, 1,2, 3, 4.5, 6, 12, 24, 48 and 72 hours for samples after both cold (c) temperatures of 2°C and ambient (a) temperatures of 17.5°C. From each of the three *Vaccinium* species, *V. myrtillus* (M) graphs A and B and D, *V. vitis-idaea* (I) graphs D and E. *V. uliginosum* samples were also tested but the expression levels were undetectable. Samples are normalised to the 0h ambient control for their respective species. Error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Student's *t*-test.

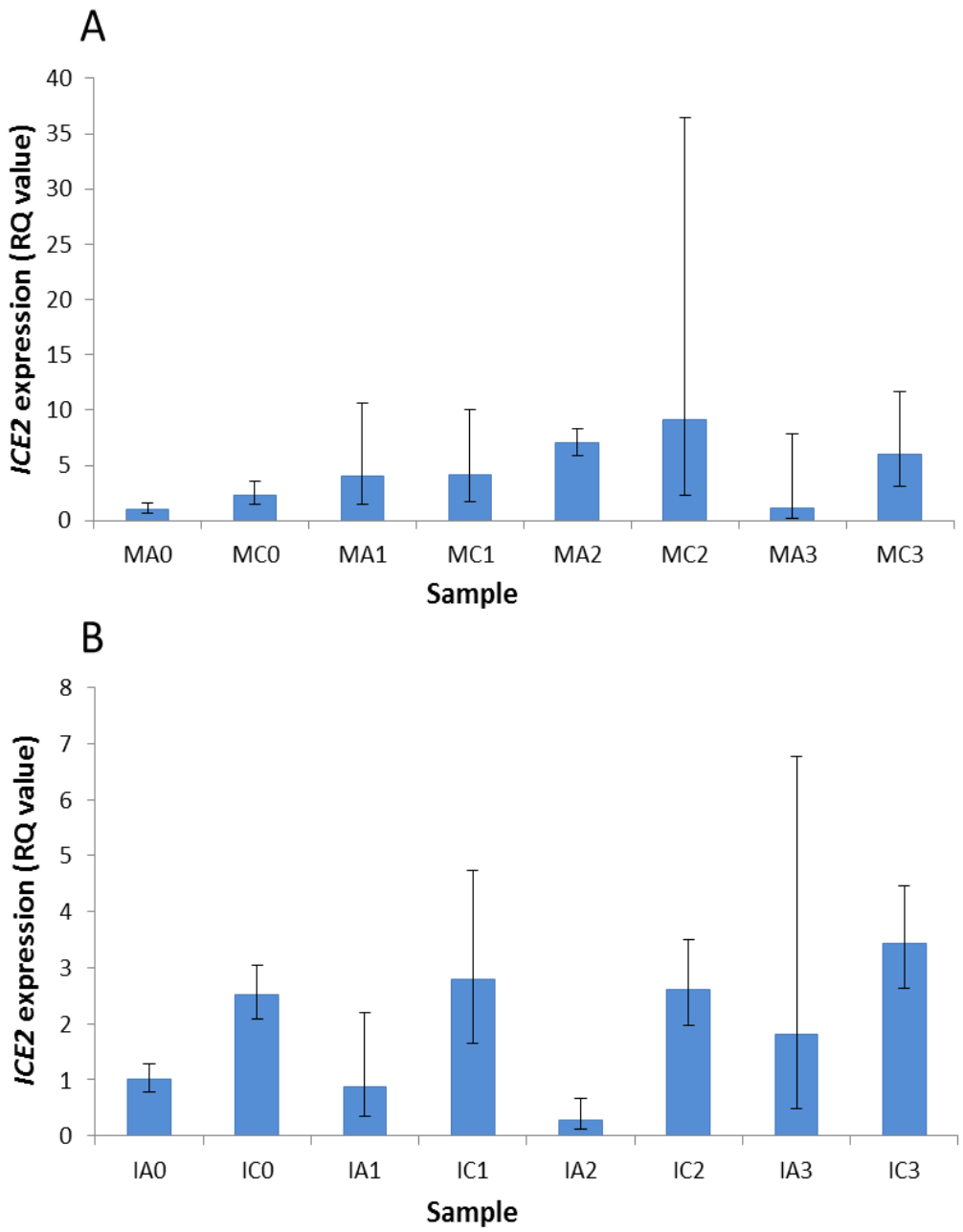


Figure 6-14: qPCR results for *ICE2* expression with 5 replicate wells for each sample. Time points 0, 1, 2, 3h for samples after both cold (c) temperatures of 2°C and ambient (a) temperatures of 17.5°C. From *V. myrtillus* ((M) graph A) and *V. vitis-idaea* ((I) graph B). Samples are normalised to the 0h ambient control for their respective species. Error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Students *t*-test.

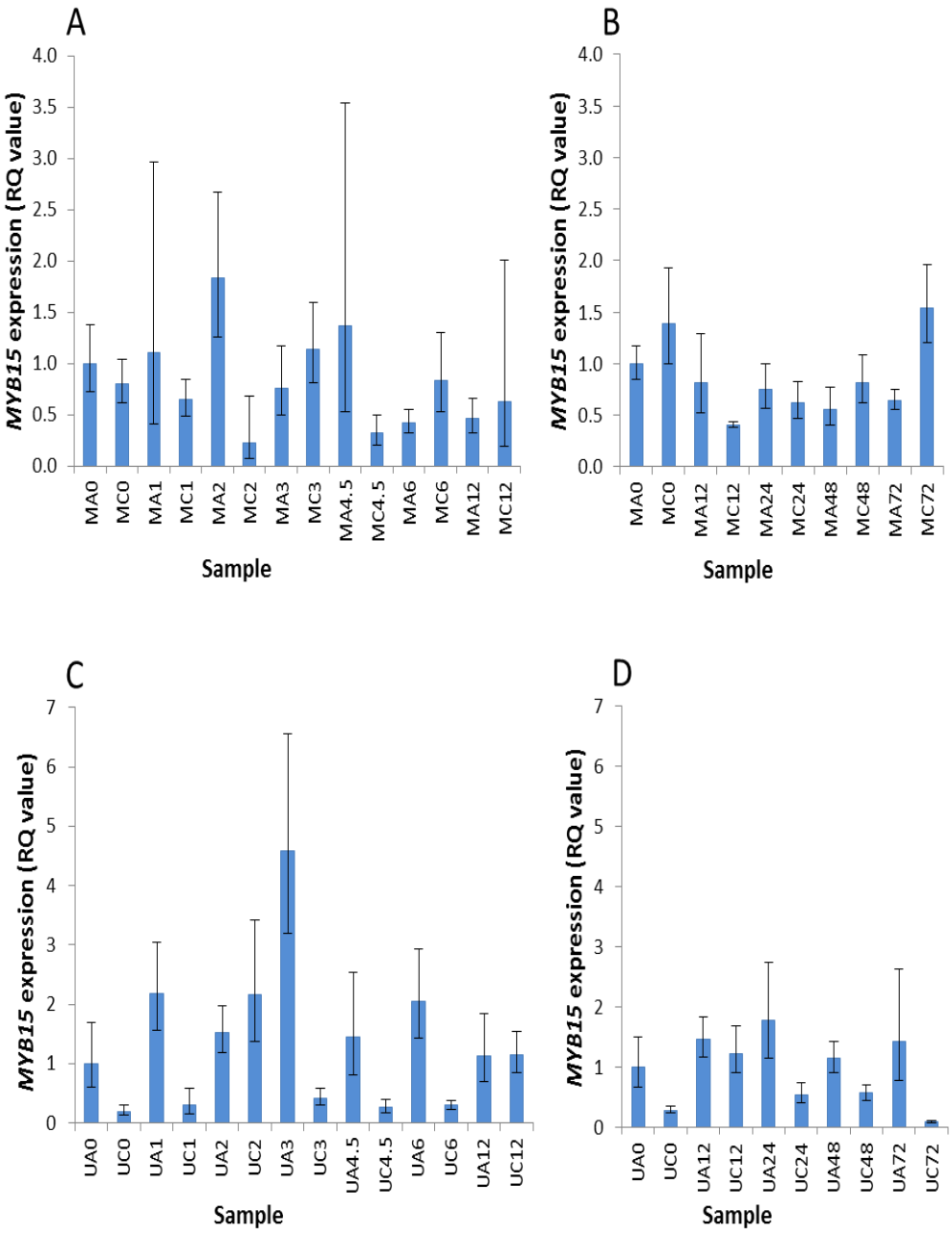
6.3.4.2 MYB15

MYB15 is a negative regulator of *CBF/DREB1;* however, it is still not known at which level this regulation occurs (Agarwal et al., 2006). In *Arabidopsis MYB15* has been found to be expressed constitutively in all tissues within the plant. Although *MYB15* overexpression has been shown to have a negative effect on *CBF/DREB1* expression, it has been shown that *MYB15* expression is up regulated in response to cold.

Figure 6-15 parts A and B show that *MYB15* expression in *V. myrtillus* does not change much over the whole time course as the greatest increase is only 1.5 times higher than the ambient 0h control. The cold 12h sample is significantly lower than both the cold and ambient 0h controls, as are the 6h cold treated sample and the cold treated 4.5h sample. However, these samples only vary between 0.5 to 1 times greater in expression than the 0h controls. Again the error bars are large for this sample set so it is difficult to conclude anything for certain from the results.

For *V. uliginosum* (Figure 6-15 C and D) the expression level of *MYB15* varies a lot more than in *V. myrtillus*. The cold 0h control is significantly lower than the ambient 0h control. The highest increase is the 3h ambient sample which has a 5 fold increase in expression in comparison to the 0h ambient control. The cold samples for 4.5h, 6h and 72h all have significantly lower expression than the 0h ambient control but are not significantly different to the 0h cold control.

In *V. vitis-idaea* (Figure 6-15 E and F) the expression levels of *MYB15* although varying between samples, do not have much overall change in relative expression. At 2h both ambient and cold samples have a significant decrease in expression compared to both the ambient and cold 0h controls. The 3h cold sample has a 2-fold increase in expression compared to both cold and ambient 0h controls. Both cold and ambient 12h samples have a significant decrease in expression compared to both 0h controls.


A
MYB15 expression (RQ value)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MA0
MC0
MA1
MC1
MA2
MC2
MA3
MC3
MA4.5
MC4.5
MA6
MC6
MA12
MC12
Sample
B
MYB15 expression (RQ value)
MA0
MC0
MA12
MC12
MA24
MC24
MA48
MC48
MA72
MC72
Sample
C
MYB15 expression (RQ value)
7
6
5
4
3
2
1
0
UA0
UC0
UA1
UC1
UA2
UC2
UA3
UC3
UA4.5
UC4.5
UA6
UC6
UA12
UC12
Sample
D
MYB15 expression (RQ value)
UA0
UC0
UA12
UC12
UA24
UC24
UA48
UC48
UA72
UC72
Sample

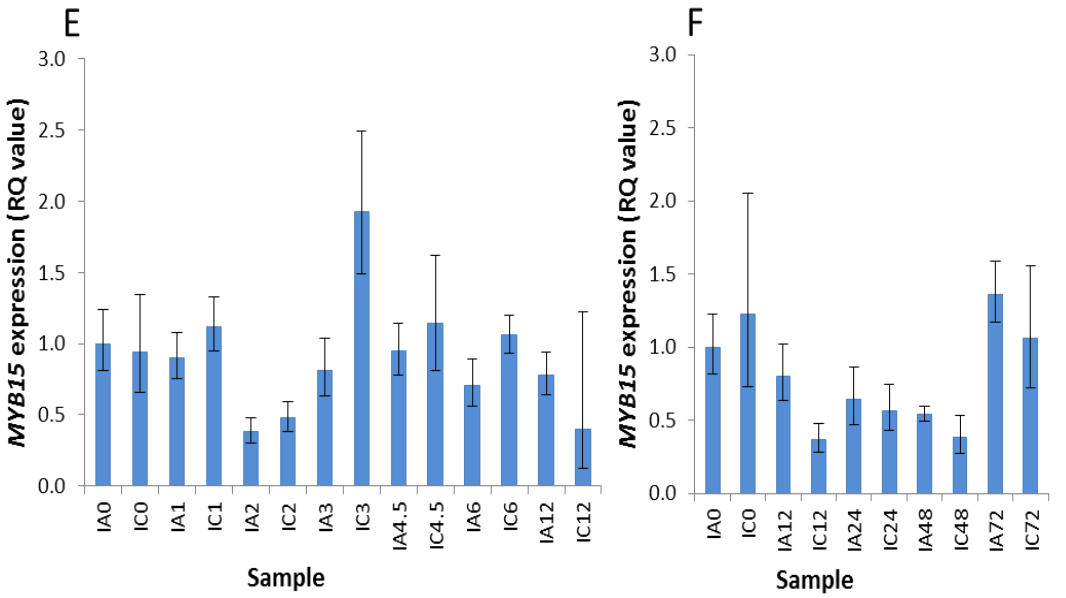


Figure 6-15: qPCR results for *MYB15* using an extended time course of time points 0, 1,2, 3, 4.5, 6, 12, 24, 48 and 72 hours for samples after both cold (c) temperatures of 2^{o}C and ambient (a) temperatures of 17.5^{o}C. From each of the three *Vaccinium* species, *V. myrtillus* (M) graphs A and B, *V. uliginosum* (U) graphs C and D, *V. vitis-idaea* (I) graphs D and E, samples are normalised to the 0h Ambient control for their respective species. Error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Student's *t*-test.

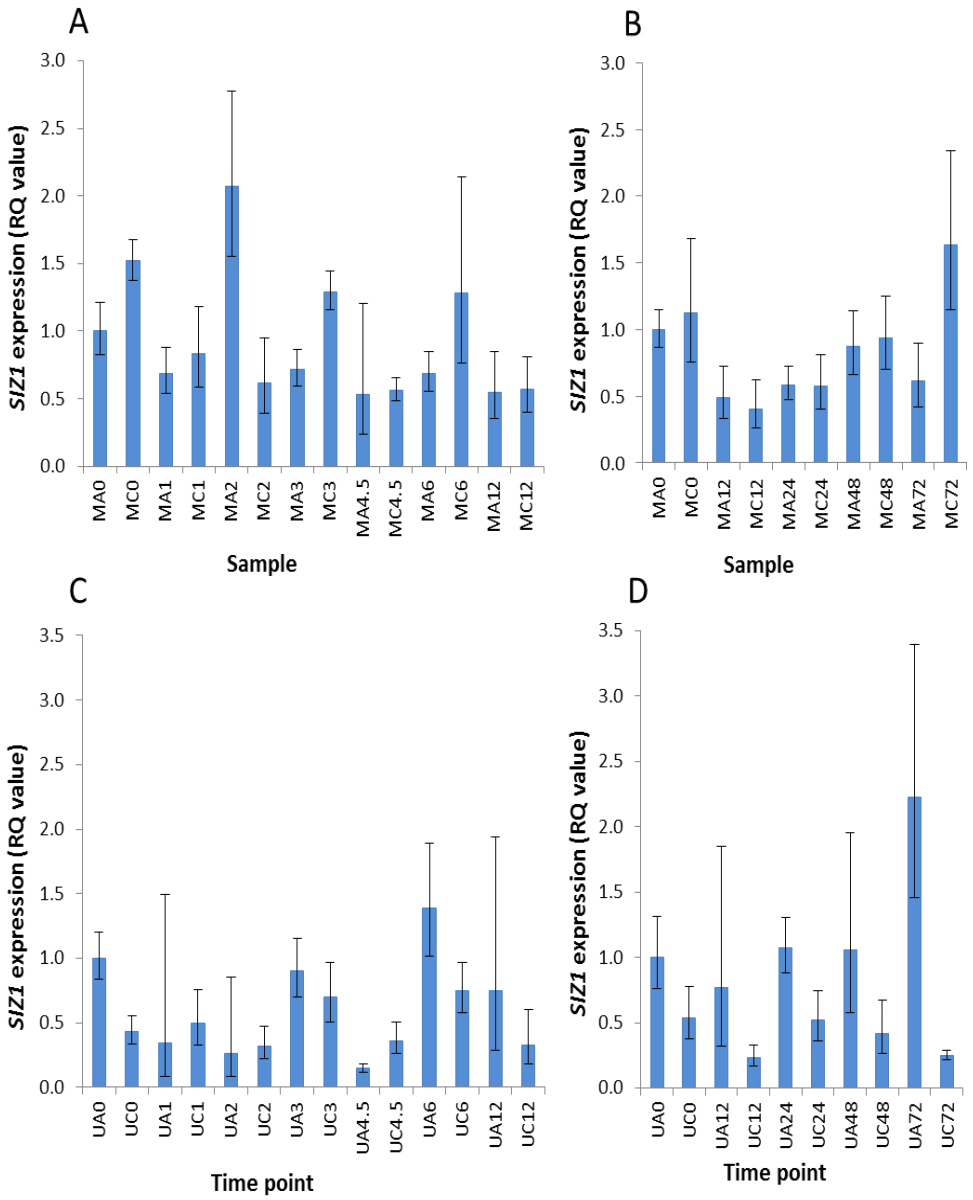
6.3.4.3 SIZ1

SIZ1 positively regulates *CBF/DREB1* expression by sumoylation, increasing the stability of ICE1/ ICE2 and therefore allowing ICE1/ICE2 to then induce increased expression of *CBF/DREB1*. SIZ1 is constitutively active in *Arabidopsis* and expression of *SIZ1* is not known to be affected by cold as transcriptional regulation is not thought directly to control SIZ1; however, the exact mechanisms of *SIZ1* activation and regulation are unknown (Miura et al., 2007).

Figure 6-16 parts A and B show that in *V. myrtillus SIZ1* expression differs between the 0h cold and ambient samples; the 0h cold sample has significantly higher *SIZ1* expression than the 0h ambient sample. The 2h ambient sample has a 2-fold increase in *SIZ1* expression compared to the ambient control and *SIZ1* expression is significantly lower after 4.5h cold then the ambient 0h control. Both ambient and cold samples at 12hours are significantly lower than the cold and ambient 0h controls.

V. uliginosum (Figure 6-16 parts C and D) also has a significant difference in expression between the cold and ambient 0h controls. The cold treated samples at 12h and 72h have significantly lower expression than the 0h cold treated control. The 72h ambient sample has significantly higher expression than the 0h ambient control. The ambient 4.5h sample also has significantly less expression than both 0h control samples.

V. vitis-idaea (Figure 6-16 parts E and F) has a clear increase in *SIZ1* expression after 1 hour exposure to cold in contrast to the majority of samples for *V. vitis-idaea* which have low expression. However, ambient samples at both 1 and 2hours show increased expression in comparison to the cold 0h control and significantly higher expression than the 0h ambient control.


A
B
C
D
SIZ1 expression (RQ value)
Sample
Time point
MA0
MC0
MA1
MC1
MA2
MC2
MA3
MC3
MA4.5
MC4.5
MA6
MC6
MA12
MC12
MA24
MC24
MA48
MC48
MA72
MC72
UA0
UC0
UA1
UC1
UA2
UC2
UA3
UC3
UA4.5
UC4.5
UA6
UC6
UA12
UC12
UA24
UC24
UA48
UC48
UA72
UC72

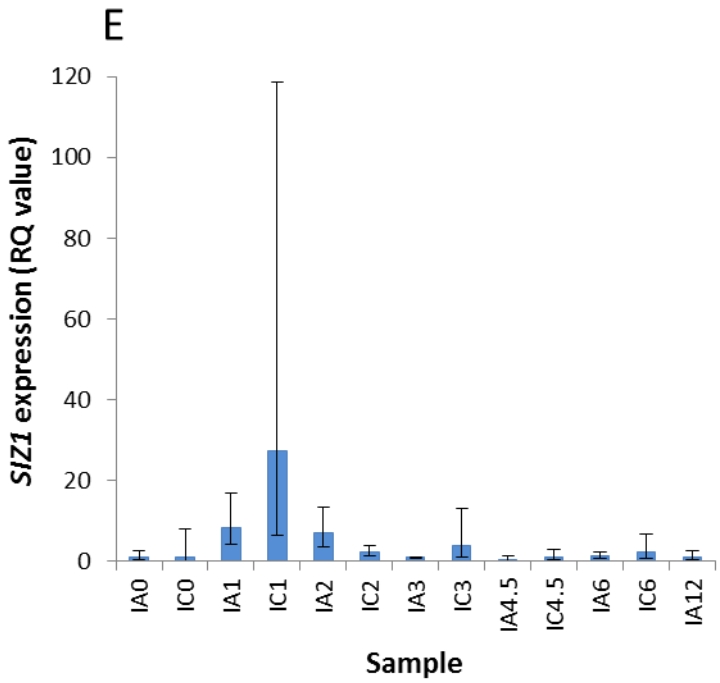


Figure 6-16: qPCR results for *SIZ1* using an extended time course of time points 0, 1,2, 3, 4.5, 6, 12, 24, 48 and 72 hours for samples after both cold (c) temperatures of 2°C and ambient (a) temperatures of 17.5°C. From each of the three *Vaccinium* species, *V. myrtillus* (M) graphs A and B, *V. uliginosum* (U) graphs C and D, *V. vitis-idaea* (I) graphs D and E, samples are normalised to the 0h Ambient control for their respective species. Error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Student's *t*-test.

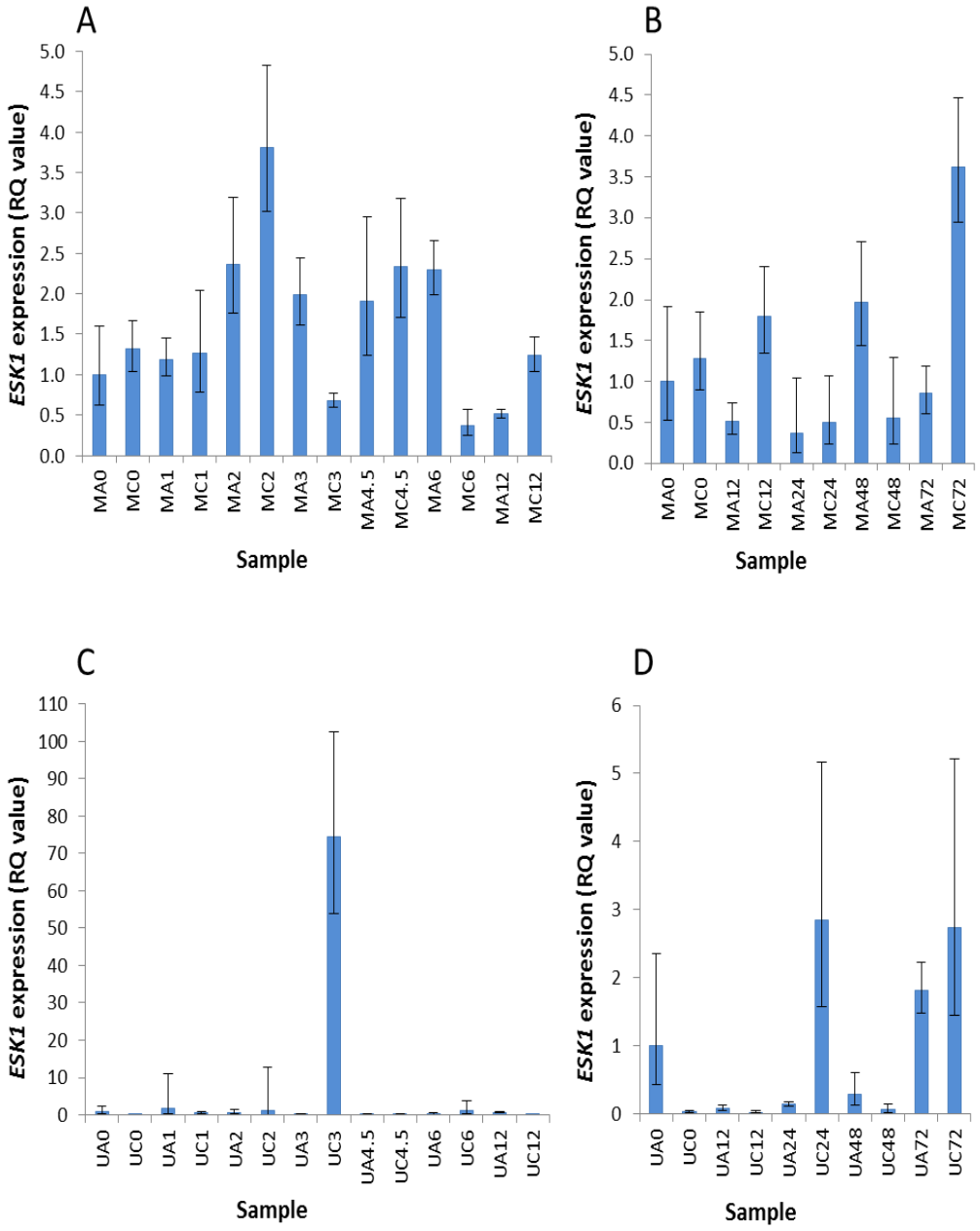
6.3.4.4 ESK1

ESK1 works independently of CBF/DREB1 (Xin et al., 2007) therefore making it a good candidate to measure as alongside *CBF/DREB1* as an alternative (Xin & Browse, 1998).

From Figure 6-17 parts A and B *V. myrtillus* has an increase in expression at both 2h time points and ambient 3h then again at both 4.5h time points and ambient 6h. Cold treated time points at 12h and 72h also have an increase in expression and ambient 48h.

V. uliginosum has a clear increase in *ESK1* expression in response to cold at 3h and then again at 24h (although the magnitude of the increase is enormously different between the two). Both 72h samples have an increased level of expression; however, it is not close in size to the increase after 3h. As there were relatively large error bars for the *V. uliginosum ESK1* samples a repeat with five replicate wells was carried out (Figure 6-18) as with some of the other genes tested. The results of this experiment are not as clear because all of the samples appear to have an increase in expression when compared to the UA0 control; however, the error bars are large on this plate. There are no large increases in expression as first suggested by the results in Figure 6-17.

For *V. vitis-idaea* (Figure 6-17 parts E and F) all of the samples except 3h and 48h cold treated have an increased expression of *ESK1* compared to the IA0 control. However, the remaining sample pairs have similar expression so both ambient and cold samples increase or decrease together.


A
ESK1 expression (RQ value)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MA0
MC0
MA1
MC1
MA2
MC2
MA3
MC3
MA4.5
MC4.5
MA6
MC6
MA12
MC12
Sample
B
ESK1 expression (RQ value)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MA0
MC0
MA12
MC12
MA24
MC24
MA48
MC48
MA72
MC72
Sample
C
ESK1 expression (RQ value)
110
100
90
80
70
60
50
40
30
20
10
0
UA0
UC0
UA1
UC1
UA2
UC2
UA3
UC3
UA4.5
UC4.5
UA6
UC6
UA12
UC12
Sample
D
ESK1 expression (RQ value)
6
5
4
3
2
1
0
UA0
UC0
UA12
UC12
UA24
UC24
UA48
UC48
UA72
UC72
Sample

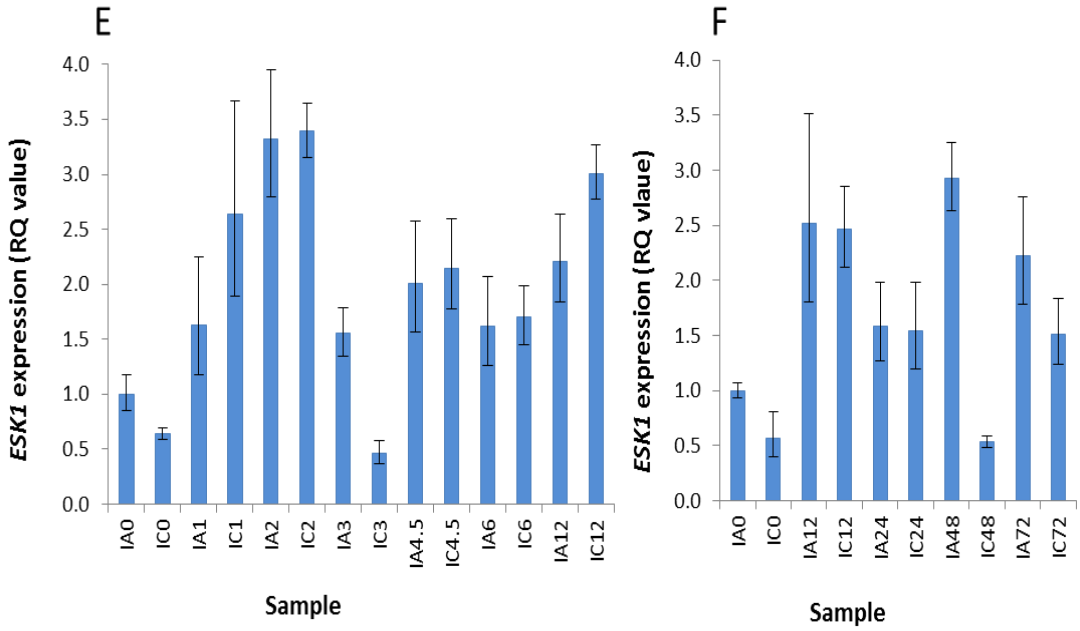


Figure 6-17: qPCR results for *ESK1* using an extended time course of time points 0, 1,2, 3, 4.5, 6, 12, 24, 48 and 72 hours for samples after both cold (c) temperatures of 2°C and ambient (a) temperatures of 17.5°C. From each of the three *Vaccinium* species, *V. myrtillus* (M) graphs A and B, *V. uliginosum* (U) graphs C and D, *V. vitis-idaea* (I) graphs D and E, samples are normalised to the 0h Ambient control for their respective species. Error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Student's *t*-test.

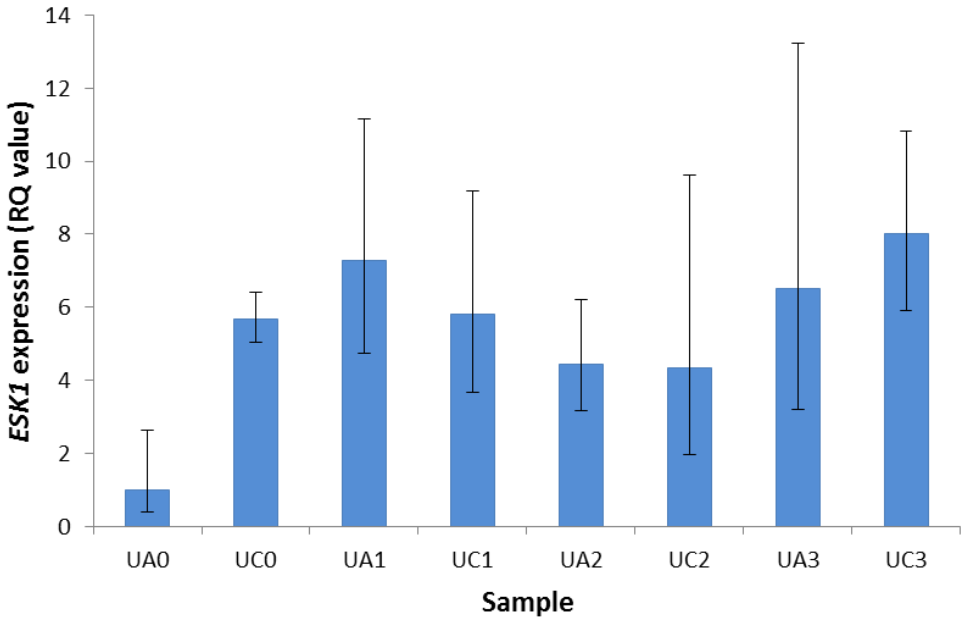


Figure 6-18: qPCR results for *ESK1* expression with 5 replicate wells for each sample. Time points 0, 1, 2, 3h for samples after both cold (c) temperatures of 2^{o}C and ambient (a) temperatures of 17.5^{o}C, from *V. uliginosum* (U). Samples are normalised to the 0h ambient control for their respective species. Error bars represent RQ_{min} and RQ_{max} with a 95% confidence level using the Students *t*-test.

6.4 Discussion

6.4.1 *Vaccinium* time course experiments

The research described in this chapter was performed in order to determine whether the three Arctic *Vaccinium* species display an increase in *CBF/ DRBE1* expression in response to cold. As part of this, the experiments evolved into a number of time course experiments.

The first challenge was extracting RNA from three species which could be frozen in liquid nitrogen but were still not friable. After being frozen the ground up tissue oxidised and turned black due to the high levels of phenolics present (Stewart & Bannister, 1973). Such phenolics are likely to interfere with any reverse transcription and subsequent qPCR reactions so an adapted CTAB method for *V. myrtillus* berries was used (Jaakola et al., 2001). For early RNA extractions some of the contaminants were still present in the samples which could give variable results; the method was optimised progressively so that the 2013 samples were as clean as possible.

The two hour time point was chosen for the first cold treatment experiment (2010) as in *Arabidopsis* the highest level of *CBF/DREB1* expression is after 2h in the cold (Gilmour et al., 1998, Liu et al., 1998). This made it a logical starting point for *Vaccinium* as even if the response was faster than two hours there should still be some change in expression noticeable after 2h (assuming the expression is a curve in expression like *Arabidopsis* rather than a sharp spike). This should also be the case if the change in expression took longer than 2h to occur. The first cold treatments used wet ice to cold treat the tissue samples which meant that although there was a change in *CBF/ DREB1* expression as a response in all three *Vaccinium* species the temperature was not accurately recorded and the samples were in the dark.

The 2010 time course showed the need for a broader range of time points to establish whether the response measured is the maximum *CBF/DREB1* response of all three species or whether the 2h time point had caught either side of an increase in *CBF/DREB1*. It was possible that the increase in expression seen at 2h was only the beginning of a much larger *CBF/DREB1* response or the point after a large increase in CBF/DREB1 increase where the expression levels are decreasing. It was obvious that a much larger scale time course was needed with a broad range of time points; however, this was not easy to arrange. The plants in Abisko have a short growth season (as described in Chapter 1) and are not easily accessible from the UK. The 2011 time course was arranged so that samples were collected mid to late August 2011 when samples from each of the 3 species were to be collected and brought back in under 24h to the UK then transferred to identical Arctic growth cabinets

which were pre-programmed from Abisko to match the exact temperature and light conditions in which the plants were growing (see Table 6.6).

The plants would then have had time to recover from the non-controlled high temperatures endured whilst travelling, before starting the cold treatment time course. The plants were split in half with half of each species in each of the two growth chambers. A temperature of 1^{o}C was chosen to attempt to trigger acclimation within the plants without actually freezing them. Avoiding freezing the plants became more of a priority, as import laws made it essential to root wash the plants before bringing them back the UK. Temperatures of below 1^{o}C would freeze the beakers of water the plants were in causing the roots to freeze. Under normal conditions the leaves and above ground tissue would freeze first and then after a prolonged period of low temperatures the roots and ground would slowly begin to freeze. If the roots of the *Vaccinium* began to freeze it would result in an entirely different freezing stress time course rather than looking at the expression of *CBF/DREB1* during acclimation. However, a slightly lower temperature of 0^{o}C or a time course over a range of different cold temperatures would have been preferable as it would allow the optimal temperature to trigger a *CBF/DREB1* response to be found.

The 2011 time course is most likely to have not been ideal as both the temperatures in Abisko around the time the samples were collected, and the cold temperatures used to trigger acclimation in the plants were not optimal. The weather in Abisko whilst the samples were collected had begun to change and night temperatures had begun to drop. This was having an effect on the plants as during the week the *Betula spp.* went from having full leaves to having few suggesting plants were initiating programs preparing for winter. It is very likely that the *Vaccinium* samples had thus already acclimated before they came back to the UK so an acclimation treatment of 1^{o}C would not induce an acclimation response once already acclimated.

The 2012 time course was planned for much earlier in the year around midsummer (late June –July). However, after an unusually late snowfall, the snow had only just melted the week before the samples were collected meaning that there were some patches of snow still around in low areas. Combined with a mild winter (2011-2012) there was also the problem of a bad outbreak of *Eppirita autumnata* (Jepsen et al., 2008) which in a few days had destroyed the majority of the newly formed birch leaves and a lot of the *V. myrtillus* and *V. uliginosum*. These two combined factors meant that the samples were almost certainly stressed before they were brought back to the UK, this was further combined with delays when returning to the UK causing the plants to be at high temperatures (~30^{o}C) for

prolonged periods with very little water. The combined problems meant that the RNA extracted from the samples had degraded.

As the previous two years had a number of problems transporting plants back and treating them due to how they were transported etc. it was decided that in 2013 the cold treatments and RNA extraction would be carried out in Umeå so that just the extracted RNA had to be transported. Although the cold treatments and RNA extractions appear to have been successful to an extent, the results show the need for more biological replicates and a broader range of temperatures. *V. uliginosum,* in contrast with the other two *Vaccinium* species, has highest expression of *CBF/DREB1* at ambient temperatures rather than cold, suggesting that different temperatures need to be tried with lower ambient and freezing temperatures to see if the levels of *CBF/DREB1* in ambient *V. uliginosum* samples remain high. Also, once the ideal time point for each species was identified a shorter more detailed time course over that period would give a clearer picture of exactly what happens in response to cold for example a time point every 30 minutes over a 3h to 4h window.

Ideally if there was a way to set this experiment up in the field it would be better as the other factor that changes is the location of the 3 species. This could be done either at the Vindelns fieldstation or Abisko research station. In an ideal world the best time to carry out this experiment would be as the temperatures begin to decrease in autumn with the samples processed at the field station. However this would be difficult to carry out and would require precise timing which may be difficult to achieve. An alternative would be to cold treat samples *in situ* in the forest or tundra and take samples to process.

V. uliginosum in Umeå is found under trees to shade it so it is possible that the direct light was also causing a stress response. It is also impossible to dig up patches of plants without damaging the roots of some plants. The plants around the edges of each patch were avoided for this reason; however, *V. uliginosum* in particular have very deep roots which were certainly damaged when the plants were dug up. When the samples for this experiment were collected two sets of samples were collected for each species and treatment at each time point. The second set however were not processed and remain in a freezer in Umeå due to the short window of time to carry out experiments in Umeå and the large amount of time it takes to carry out each RNA extraction. The error bars throughout the real time data suggest that at least one further biological replicate and more technical replicates are needed to conclude many of the results for certain.

6.4.2 Non-CBF/DREB1 proteins associated with cold

Isolating and sequencing the non-*CBF/DREB1* genes associated with cold was difficult as there is currently no way to be 100% certain that the right genes have been isolated without a full genome sequence. When the *V. corymbosum* genome is assembled it will be possible to compare the sequences from other *Vaccinium* species. However, the sequences of the genes used have been checked in as many ways as possible (and using the *V. corymbosum* scaffolds which will eventually be used to construct the full genome) to provide the highest likelihood that the correct genes were identified.

Not having a full genome sequence also caused problems for the design of primers to amplify these genes, qPCR and even the RACE PCR as it is possible that the primers used will bind to multiple sites. Priming of multiple binding sites could potentially be why the RACE PCR reactions were unsuccessful. Another factor affecting the RACE PCR reactions was also potentially the purity of the extracted RNA used. The RNA used for the cDNA synthesis as part of RACE PCR, was from one of the first sets of RNA extractions carried out and may not have been of as good quality as later extractions. (Later extractions were further optimized to contain less impurities and a higher concentration of RNA).

Multiple copies of some genes were amplified from the primers designed, this became apparent from the sequencing results. The target genes were amplified, gel extracted, and sequenced which showed that multiple sequences were present together in the same reaction. As the fragments for cloning were produced in the same way, it is possible that different copies of each gene were present in the colonies selected for sequencing. This was caused due to multiple copies of the same gene being present and all the same size so gel extractions contained a mixture of products. As only two colonies per reaction were isolated and sequenced it is possible that different copies of the same gene were sequenced eg. Any one of 4 genes of *SIZ1* were possible. This would possibly account for the large differences in sequence present between species for some genes. In particular this is most likely for SIZ1 as there were at least 4 copies of the gene that were sequenced at the same time (Figure 6-6).

6.4.3 Testing expression of Non-*CBF/DREB1* genes

The final aim of this chapter was to test the expression of other genes associated with freezing tolerance in *Vaccinium*. This was completed but again showed the importance of more biological replicates for these experiments. The statistical error in each set of results shows that the experiments need repeating with more technical replicates and new

biological replicate samples (as discussed above). More technical repeats were not possible due to a lack of RNA. Each RNA extraction has a maximum concentration of around 300ng/µl in 50µl. To make sure that some sample returned to the UK from Umeå safely, half of the RNA extracted was left in Umeå as a backup in case the first samples shipped to the UK degraded in transit. The cDNA used for qPCR has a maximum limit to the number of times it can be freeze-thawed before it becomes unusable. This meant that after the primers and DNase/ cDNA synthesis had been optimized there was enough RNA to make enough cDNA to run one aPCR plate for each set of primers with a little spare. The remaining cDNA was used to repeat qPCR plates that hadn't worked first and to carry out the qPCR experiments with five technical replicates. Some of the plates were set up multiple times such as *V. uliginosum CBF/DREB1* and *ICE2* as they didn't work well or at all. Multiple repeats had to be carried out for these genes to ensure this was due to a low level of expression rather than a technical error. As far as possible the samples were collected from the same plants; however, this required a large number of leaves so a number of plants was used, especially for *V. vitis-idaea.* As *V. vitis-idaea* only has one small above ground shoot, it was impossible to determine whether samples collected were from the same individual plant or a combination of a number of plants. This would introduce variation in expression levels between different samples.

As the expression levels for each gene vary between species and appear to behave in different ways in response to the same environmental cue it is more logical to look at the gene expression for each species in terms of the whole CBF/DREB1 pathway rather than comparing each gene on an individual basis. However, this can only be a speculative conclusion, as little is known about the *Vaccinium* genome in general. It is not known how many copies of the *CBF/DREB1* genes are present in the *Vaccinium* genome or if the function of these genes is the same as in *Arabidopsis*. The experiments performed work on the assumption that the CBF/DREB1 pathway and system work in the same way as *Arabidopsis*; however, even in *Arabidopsis* some functions of genes and proteins in this pathway are still under question. Certain proteins in the CBF/DREB1 pathway, such as ICE1, have been shown to vary in activation and function between species and little is known about their transcript levels. It is likely that the *Vaccinium* species used also deviate slightly from the *Arabidopsis* model.

In *Arabidopsis*, *ICE1* expression (Chinnusamy *et al.*, 2003b, Fursova *et al.*, 2009) is thought to be constitutively active throughout all tissues in the plant. *SIZ1* is also thought to be constitutively active (Miura & Ohta, 2010) and its transcriptional regulation is not thought

to be altered by cold; however, the mechanism through which SIZ1 is activated is unknown (Miura *et al.*, 2005). Interestingly, although *MYB15* is also thought to be constitutively expressed and is a negative regulator of *CBF/DREB1,* the expression levels of *MYB15* increase in response to cold in *Arabidopsis* (Agarwal *et al.*, 2006), possibly highlighting a function in a non-*CBF/DREB1* cold response pathway.

In ambient conditions HOS1 (an E3 ubiquitin ligase) targets ICE for degradation (Lee et al., 2001). MYB15 binds to the MYB recognition sequence in the promoter of *CBF/DREB1* negatively regulating its expression (Ishitani et al., 1998).

Under cold conditions it is thought that SIZ1 sumoylates ICE (Miura *et al.*, 2007), improving its stability and therefore allowing ICE to bind to the MYC recognition domain on *CBF/DREB1* causing increased expression. Sumoylated ICE is also thought to repress MYB15, preventing the negative regulation of CBF/DREB1 (Agarwal et al., 2006, Miura et al., 2007). However, there are other possible methods of ICE activation in *Arabidopsis*. Other studies of ICE activation state that ICE is constitutively active under all conditions but Chinnusamy et al., (2003a) suggest that ICE may be phosphorylated in response to cold to increase its activity.

There are many variations in the CBF/DREB1 pathway between the following study species. In *Camellia sinensis* (tea) (Wang et al., 2012), *Malus x domestica* (apple) (Feng et al., 2012) and *Eucalyptus camaldulensis* (Eucalyptus) (Lin et al., 2014) it was shown that there is the same level of *ICE* expression present in both cold and ambient samples. However, in *Vitis vinifera* (grape) *spp*. (Annette, 2014), *Lycopersicon esculentum* (tomato) (Feng et al., 2013) and *Oryza sativa* (rice) (Nakamura et al., 2011) the level of ICE expression has been shown to change in response to cold. In *V. vinifera* it has been shown that there are four *ICE* genes present which can undergo alternative splicing to produce seven ICE splice variants which fulfil different functions in response to abiotic stress (Rahman, 2014). In *Arabidopsis* ICE1 has most effect on the expression of CBF3 (Chinnusamy et al., 2003a) and ICE2 affects the expression of CBF1 (Fursova et al., 2009), whereas in *Malus x domestica* ICE1 can bind CBF2 (Feng et al., 2012) and in *Eucalyptus* ICE1 binds CBF1 and CBF3 (Lin et al., 2014). In some woody perennials such as citrus, *Populus euphratica* (poplar) and *Eucalyptus camaldulensis* (eucalyptus) there are only one or two ICE genes present in their genomes (Gusta & Wisniewski, 2013).

The exact mechanism of activation of *CBF/DREB1* genes also differs in some species other than *Arabidopsis.* In *O. sativa* (Nakamura et al., 2011), *T. aestivum* (Badawi et al., 2008) and *P. euphratica* (Li et al., 2012) *CBF/DREB* genes undergo alternative splicing as

transcriptional regulation in response to temperature changes; however, the *CBF/DREB1* identified from *Vaccinium* species does not have an intron so this is unlikely.

6.4.4 *V. myrtillus*

V. myrtillus displays relatively high levels of *CBF/DREB1* expression after two hours exposure to cold (Figure 6-1 and Figure 6-2), (this was shown in both the 2010 and 2013 experiments). However, the results from the *ICE2* qPCR suggest that there is no change in expression of *ICE2* in association with this. In some species *ICE* is constitutively expressed and the protein produced is modified accordingly (Annette, 2014) which could be what is happening in *V. myrtillus*. There was a significant decrease in expression level in the cold treated samples at 1h and 6h and both ambient and cold 0h controls; however, the overall magnitude of the increases and decreases across all of the samples suggest that *ICE2* is constitutively expressed in *V. myrtillus*. *MYB15* and *SIZ1* also show few significant differences in the magnitude of increase or decrease in expression levels suggesting that both *MYB15* and *SIZ1* are also constitutively expressed. Separate to the CBF/DREB1 pathway, the expression pattern for *ESK1* in *V. myrtillus* also showed an increase in response to cold after 2h and 72h; however, there are also slightly increased levels in many of the ambient samples suggesting that there is no correlation between the temperature and results obtained. The combined results suggest that *V. myrtillus* has a CBF/DREB1 pathway similar to *Arabidopsis* as the highest increase in *CBF/DREB1* expression is after 2h and the other genes involved in the CBF/DREB1 pathway appear to be constitutively expressed with little difference between the cold and ambient samples over the duration of the time course.

6.4.5 *V. uliginosum*

V. uliginosum CBF/DREB1 displays consistent relative increases in expression in response to the ambient 16^{o}C temperature rather than the cold treatment. It is possible that the cold treatments did cause a response; however, because the ambient levels of expression are high it would be difficult to see without more repeat samples at a range of ambient temperatures. As the cold treated samples are normalised to the ambient samples, so a control at a temperature certain to have low CBF/DREB1 expression would be a better control. There is also a possibility that *CBF/DREB1* expression decreases in response to cold in *V. uliginosum;* however, this is inconsistent with the results from the 2010 samples. *V. uliginosum* undergoes the most damage when being moved to the growth chambers so it is possible that it suffered drought stress in the ambient room as the temperatures were higher. Combined with damaged roots, this may mean that it was unable to maintain

enough water to prevent drought stress occurring. At colder temperatures there would not be as much transpiration occurring and lower temperatures thus would reduce the level of drought stress incurred. It is not known whether *Vaccinium* has multiple *CBF/DREB1* genes like *Arabidopsis;* if more than one *CBF/DREB1* is present in *Vaccinium* it cannot be said for certain which *CBF/DREB1* is being measured, therefore there is no way of knowing whether the results show the expression of a *CBF/DREB1* gene such as *CBF4* in *Arabidopsis,* (Haake *et al.*, 2002) which responds to heat/drought rather than cold. If multiple CBF/DREB1 genes are present in *Vaccinium*, designing primers to specific *CBF/DREB1* genes that are associated with cold would be a way to avoid this problem.

ICE2 expression was undetectable in *V. uliginosum,* either due to there being no expression or levels too low to detect. *MYB15,* however, showed a decrease in expression after cold treatment and appeared to have higher levels of expression at 16^{o}C. Following the pattern of *MYB15* expression in *Arabidopsis,* this would suggest that *V. uliginosum* has more of a response from the CBF/DREB1 pathway at 16^{o}C than 1^{o}C. Alternatively it could be that CBF/DREB1 has reduced inhibition by MYB15 in response to cold as a result of less MYB15 production. However, this conflicts with the expression data for *CBF/DREB1* as it would be expected that if this were the case, the results would show induction of *CBF/DREB1* at the cold treated time points, due to less inhibition of ICE1. *SIZ1* shows little change in expression between the different time points with significant decreases in expression between some samples; however, the overall decrease in expression between any samples is low. This fits with the pattern of *SIZ1* expression in *Arabidopsis* and the suggestion that *SIZ1* expression is not affected by cold. A repeat of the time course with a range of temperatures is needed before any firm conclusions can be reached.

The current *Vaccinium CBF/DREB1* primers are designed to regions that are conserved between the three species. In *Arabidopsis* there are regions that are highly conserved between all four *CBF/DREB1* genes (Thomashow et al., 2001); the *Vaccinium* CBF/DREB1 qPCR primers amplify one of the conserved regions within the hydrophobic domains of the CBF/DREB1. It is therefore possible that the primers have unintentionally been designed so that they measure combined expression of total *CBF/DREB* genes rather than just one. Alternatively, the results of the *ESK1* qPCR (Figure 6-17) show high levels of expression in response to cold at 2h, which suggest that *V. uliginosum* may use *ESK1* in response to cold instead of *CBF/DREB1*. However, because the results from the five replicate qPCRs conflict with this, further work is needed before a definite conclusion can be reached.

6.4.6 *V. vitis-idaea*

V. vitis-daea displays a clear peak in *CBF/DREB1* expression after 1h of exposure to cold; there is also a peak in *ICE2* expression at the same time point, although this conflicts with the idea that *ICE* genes are constitutively expressed in *Arabidopsis*. However it is also possible that the peak in expression of ICE2 at 1h cold is an artefact caused by high CT values for the housekeeping gene used. Further repeats need to be carried out to confirm whether this is a peak in expression. *MYB15* expression changes very little in response to any of the treatments showing *MYB15* expression does not increase in response to cold. *SIZ1* however displays a large increase in expression after 1h exposure to cold. The combined results show that the CBF/DREB1 pathway in *V. vitis-idaea* is not the same as in *Arabidopsis* for *ICE2*, *MYB15* and *SIZ1*. The result for *SIZ1* is particularly different as, in *Arabidopsis SIZ1* has not previously been shown to have a response to cold at the gene expression level. The *ESK1* results for *V. vitis-idaea* in response to cold are the same for both cold and ambient samples except for at 3h and 72h where the cold treated sample for both has lower expression than the ambient.

7 Chapter 7 - General discussion and further work

7.1 Discussion

7.1.1 *CBF/DREB1* shows a high level of sequence similarity between *Vaccinium* species

Due to the differences in winter survival strategies of the three Arctic *Vaccinium* species and different favoured growth habitats it was possible that there would be differences between their encoded protein sequences of *CBF/DREB1s*. All three species were found to have a high level of sequence similarity and are close in sequence to *V. corymbosum*. It was possible to identify regions highlighted as important in *Arabidopsis* in each of the three *Vaccinium* sequences. Regions such as the nuclear localisation sequence (Stockinger *et al.*, 1997)(Figure 3-1), the *COR* gene binding domain (Canella et al., 2010) (Figure 3-1), and the AP2 signature sequence (Canella et al., 2010, Gilmour et al., 1998, Riechmann & Meyerowitz, 1998, Stockinger et al., 1997) (Figure 3-1) were conserved between *Vaccinium* species, and between *Vaccinium* and *Arabidopsis,* with relatively few changes. However, the sequence at the C-terminal end was not as well conserved with only two of the six hydrophobic domains from *Arabidopsis* (Wang et al., 2005) identifiable in any of the *Vaccinium* species.

Although the protein sequences of the three *Vaccinium CBF/DREB1s* are very similar (Figure 3-1), when over-expressed in *Arabidopsis* they behaved differently. In Chapters 3 and 4 the behaviour of the three *Vaccinium CBF/DREB1s* was characterised by also measuring the level of each *Vaccinium CBF/DREB1* expressed in each line and then comparing the levels of *COR* gene expression this induced in each case. The average rosette diameter for each plant was measured to determine an average size for each line and give an indication of any dwarf phenotype (discussed below).

From the results of the *Vaccinium* expression time course experiments (Chapter 6) it appears each *CBF/DREB1* gene may behave differently in their respective *Vaccinium* species. It is possible that this is due to differences between species in other factors involved in regulating the CBF/DREB1 pathway, such as the activation of *CBF/DREB1* expression by ICE1 (Chinnusamy et al., 2003) or the repression of *CBF/DREB1* expression by MYB15 (Agarwal et al., 2006). However, further time course experiments and expression studies are needed before firm conclusions can be reached due to the variability of the data obtained.

7.1.2 CBF/DREB1 from *V. myrtillus* can induce freezing tolerance in *Arabidopsis*

The characterisation of the three *Vaccinium CBF/DREB1* genes in *Arabidopsis* showed that *V. myrtillus CBF/DREB1,* when overexpressed in *Arabidopsis,* could induce freezing tolerance without a period of acclimation. Transgenic *Arabidopsis* lines showed high levels of expression of all three *Vaccinium CBF/DREB1s* in *Arabidopsis* but only *V. myrtillus* lines showed increased expression of *COR* genes in *Arabidopsis*. The average rosette diameter for the *V. myrtillus* lines was also significantly smaller than the *V. uliginosum*, *V. vitis-idaea* and wt *Arabidopsis* lines. Overexpression of functional *CBF/DREB1s* and other AP2 transcription factors in *Arabidopsis* has been shown to cause dwarfism and delayed flowering time due to their role in repressing giberellic acid (GA) biosynthesis (Magome et al., 2004). Under normal conditions GA regulates the production of DELLA proteins. As the level of bioactive GA was reduced by the overexpression of *V. myrtillus CBF/DREB1*, DELLA proteins were not inhibited (Niu et al., 2014), therefore allowing stress response pathways to be promoted, causing pathways responsible for processes such as growth and flowering time to be reduced (Achard et al., 2008).

Freezing assays were carried out to confirm whether the increase in *COR* gene expression induced by the *V. myrtillus CBF/DREB1* had also induced freezing tolerance in the transgenic lines. The results showed that the *V. myrtillus* lines were able to survive freezing at -7^{o}C without a period of acclimation, whereas the wt, *V. uliginosum* and *V. vitis-idaea Arabidopsis* lines were unable to survive. The difference in ability to induce freezing tolerance was further investigated using transient expression of the three *Vaccinium CBF/DREB1s* in *Nicotiana benthamiana* (tobacco) to characterise nuclear-localisation and stability. Using GFP-CBF/DREB1 fusion constructs, each *Vaccinium* CBF/DREB1 was imaged using confocal microscopy. The results showed that all three CBF/DREB1 fusion proteins were nuclear-localised, so this was not the reason for the difference in induced freezing tolerance. Western blot analysis of protein extracted from infiltrated *N. benthamiana* to detect the GFP-CBF/DREB1 fusion protein showed clear bands for *V. myrtillus* and *V. vitis-idaea* at 48h after infiltration, but no bands were detectable for *V. uliginosum* at any of the three chosen time points suggesting that *V. uliginosum* CBF/DREB1 is a less stable protein than those from the other two species.

Reporter gene assays were carried out involving transient expression of *Vaccinium CBF/DREB1* and a CRT/DRE::LUC construct co-infiltrated to establish whether each of the three *Vaccinium CBF/DREB1* proteins had the same ability to bind and activate *COR* gene expression. The CBF/DREB1 from *V. myrtillus* had a better ability to bind and induce expression via CRT/DRE compared to *V. uliginosum* and *V. vitis-idaea*. The differences

between CBF/DREB1s from the three *Vaccinium* species in ability to induce freezing tolerance in *Arabidopsis* are therefore most likely to be due to both the possible instability of the *V. uliginosum* CBF/DREB1 protein and the limited ability of *V. vitis-idaea* to bind and induce expression of *COR* genes. The instability of the *V. uliginosum* CBF/DREB1 could possibly be due to a double lysine present in the *COR* gene binding domain region (Canella et al., 2010), (which is not present in the other species of *Vaccinium)* acting as a ubiquitination site.

7.1.3 Site-directed mutagenesis of *Vaccinium CBF/DREB1* can significantly improve *COR* gene binding and activation

The *COR* gene binding domain region of *V. uliginosum* contains a double lysine (Figure 4-5) which is not present in any of the other *Vaccinium* species. This double lysine could be a potential ubiquitination site (Pickart, 2001) rendering the *V. uliginosum* CBF/DREB1 protein more unstable (as suggested above). However, altered *COR* gene binding ability may also be due to an altered shape caused by the substitution of an aspartic acid and arginine (a positive and a negative charge) present in the other species, to a double lysine (double positive charge) therefore altering the charge and potentially the shape of the protein produced. Further reporter gene assays were carried out using wt *Vaccinium* CBF/DREB1 constructs as controls for *Vaccinium* CBF/DREB1 site-directed mutagenesis constructs. Systematic mutations of amino acids that differed within the *COR* gene binding domain of the three species showed that the substitution of the double lysine to the aspartic acid, arginine sequence found in *V. myrtillus* and *V. vitis-idaea* significantly improved the *COR* gene binding and activation induced by the CBF/DREB1 from *V. uliginosum*. This improvement could have potential implications for improvement of *Vaccinium* crops such as commercial blueberry (*V. corymbosum*) which has a glycine, arginine sequence instead of the aspartic acid, arginine sequence However, further experiments are needed to investigate this fully.

To further understand the impact this substitution could have in *V. corymbosum* further characterisation of the wt *V. corymbosum* CBF/DREB1 would be needed by overexpression in *Arabidopsis* and comparison of *COR* gene expression, phenotype and freezing tolerance to the three Arctic *Vaccinium* species. Characterisation of ability to bind and activate the CRT/DRE element using reporter gene assays and western blot analysis of relative levels of CBF/DREB1 protein would also be needed to establish how the wt *V. corymbosum* behaves. Site directed mutagenesis could then be used to test the hypothesis that mutating the glycine, arginine to the aspartic acid, arginine sequence found in *V. myrtillus* may improve

freezing tolerance. Stable transformant *Arabidopsis* lines could then be produced to repeat the characterisation experiments in comparison to the wt CBF/DREB1. This could also be further tested by the production of transgenic *Vaccinium* lines (Figure 4-5).

7.1.4 There are possible similarities between the *Vaccinium* CBF/DREB1 pathways and *Arabidopsis*

It was possible to identify a number of putative components of the CBF/DREB1 pathway in *Vaccinium* by using protein sequences from *Arabidopsis* showing there is a degree of sequence conservation between the pathways. It was also possible to identify a number of different upstream components of this pathway such as *ICE2*, *SIZ1* and *MYB15* as well as CBF/DREB1-independent *ESK1*. However it was not possible to identify *HOS1* or *HOS9* suggesting that they either have substantially different sequences to *Arabidopsis* or that they are not present in *Vaccinium*. *V. vitis-idaea* appears to have the most similar response to *Arabidopsis* in terms of expression level of genes involved in the CBF/DREB1 pathway in response to cold however, further technical and biological replicate experiments are needed to confirm these results. To establish whether the CBF/DREB1 pathway works in the same way and with the same function as in *Arabidopsis,* further expression studies are needed to characterise the activity of each of the genes and subsequent proteins. Further characterisation of the CBF/DREB1 pathway in *Vaccinium* could then be used as to carry out a much larger scale cold time course experiment to produce an expression profile over a longer time frame.

7.2 Further Work

7.2.1 Further characterisation of CBF/DREB1 *Arabidopsis* lines

There are a number of experiments that need to be carried out to characterise further the *Vaccinium CBF/DREB1* overexpression *Arabidopsis* lines. Repeating the freezing assays with a range of temperatures starting from -2^{o}C and continuing at intervals of 1^{o}C until the plants are no longer able to survive, would determine whether the *V. myrtillus* CBF/DREB1 has actually increased the freezing tolerance of the transgenic *Arabidopsis* lines to allow them to survive lower temperatures than acclimated wt *Arabidopsis*. This may also show that the *V. myrtillus* CBF/DREB1 had only induced freezing tolerance to the level of acclimated wt *Arabidopsis* (Gilmour et al., 1988). Carrying out the same freezing assays after a period of acclimation for all of the transgenic *Arabidopsis* lines may also highlight further differences in freezing tolerance between the lines.

7.2.2 Further characterisation of mutant *Vaccinium CBF/DREB1s*

To characterise more completely the effect that each substitution induced by site directed mutagenesis had on the subsequent CBF/DREB1 proteins produced, further characterisation of the wt CBF/DREB1 needs to be carried out. Such as Western blot analysis of the wt *Vaccinium* CBF/DREB1 protein with an anti-ubiquitin antibody would show whether the possible difference in the stability of *V. uliginosum* is due to ubiquitination of CBF/DREB1. This could also be carried out using GFP pull downs of GFP-tagged protein both the mutant and wt CBF/DREB1 infiltrated leaves before probing with anti-ubiquitination antibodies from transiently expressed protein.

Stable *Arabidopsis* transformants need to be produced for each of the mutated *CBF/DREB1s* to test their level of expression, phenotype and ability to induce *COR* gene expression and therefore freezing tolerance in *Arabidopsis*. This would be performed in comparison to the wt *Vaccinium CBF/DREB1* overexpression lines to establish whether each mutation had improved their function, as suggested by the reporter gene assays. Stable transformants could also be used for Western blot analysis to compare the stability of each of the mutated CBF/DREB1s with the wt original to see if the stability of *V. uliginosum* CBF/DREB1 had improved with the substitution of the double lysine.

7.2.3 Further characterisation of the *CBF/DREB1* response in *Vaccinium*

Further characterisation of the CBF/DREB1 pathway of each *Vaccinium* species needs to be carried out to build up a more complete picture of what happens in response to cold in each species in terms of the CBF/DREB1 pathway and its components. An extended gene expression time course with a broader range of time points and temperatures could give further insight into this. An alternative much more time efficient way to carry this out would be to use transcriptomics to identify the transcripts that are upregulated in response to cold across a range of time points with corresponding ambient samples for comparison. However, without a genome sequence upregulated genes would have to be identified using sequence homology with species whose genome has already been sequenced. Identification of novel cold upregulated genes would be difficult, until any cold associated genes already known in other species are annotated in the *Vaccinium* results. Transcriptomics could also be used to establish whether *ESK1* or proteins such as HOS9 that are known to be CBF/DREB1 independent play more of a role in freezing tolerance than CBF/DREB1 in some *Vaccinium* species.

7.2.4 Transgenic *Vaccinium*

Traditionally methods of crop improvement used breeding between blueberry cultivars or related species (Rousi, 1966) improve desirable traits such as freezing tolerance (Chavez & Lyrene, 2009). More recently methods have been produced to generate transgenic blueberry (*V. corymbosum*) cultivars. This technique has been used to induce improved freezing tolerance in a freezing sensitive cultivar of blueberry by overexpressing *CBF/DREB1* from a more freezing tolerant cultivar (Walworth et al., 2012). To determine whether *CBF/DREB1* truly is important in the three species of Arctic *Vaccinium*, *CBF/DREB1* loss of function mutants could be produced using RNAi introduced by *Agrobacterium* mediated transformation to transform *Vaccinium* leaf explants (Senthil-Kumar & Mysore, 2011, Song & Sink, 2004). This would be the most effective method of producing loss of function mutants as the number of *CBF/DREB1* genes present in *Vaccinium* is unknown and some *Vaccinium* species are tetraploid. However, because *CBF/DREB1* in *Arabidopsis* has a developmental role, as well as being part of the stress response, meaning that the RNAi explants produced may not be viable. The RNAi may therefore have to be introduced under a conditional promoter such as a chemically induced promoter, allowing the plants to develop fully before inducing a loss of function of *CBF/DREB1*. The loss of function plants could then be used to test the effect *CBF/DREB1* has on freezing tolerance within each species.

Transgenic *Vaccinium* could also be used to test the effects of the mutated *CBF/DREB1* genes (discussed in Chapter 4) on freezing tolerance in their respective species. However, *Vaccinium* is a relatively slow growing species so this experiment would take several years to reach a stage where it would be possible to carry out freezing assays to test the effects fully.

7.3 Conclusions

From the results obtained it is not possible to say whether the three *Vaccinium* species contain genes that could be potential targets for crop improvement. CBF/DREB1 from *V. myrtillus* can induce constitutive freezing tolerance in *Arabidopsis;* however, this causes a dwarf phenotype so would significantly reduce the crop yield. It is possible that other genes such as *ICE* may be a better target for crop improvement; however, this needs further investigation.

Although there are differences in *CBF/DREB1* sequence between *Vaccinium* species, there are also physiological differences. It is possible that CBF/DREB1 could play a role in this by activating *COR* genes responsible for the different processes in each species. However,

CBF/DREB1 cannot be responsible for all of the contributing physiological factors, such as difference in leaf physiology between the three species. Therefore it is most likely that the hypothesised differences in freezing tolerance are due to a combination of factors as opposed to CBF/DREB1 alone. In *Arabidopsis CBF/DREB1* is active quickly in response to cold (Chinnusamy et al., 2006), this may also be the case in Arctic species and as such, there may be alternative mechanisms or pathways present in Arctic species that are not present in species from more temperate regions and are therefore, yet to be investigated.

Currently there are studies investigating freezing tolerance from a number of different angles. Some are investigating the role of *CBF/DREB1* in tropical species such as *Cocos nucifera* (Coconut) (ZiLong, 2012), or *Actinidia chinensis* (Kiwifruit) (Ma et al., 2014) to further understand why these plants are unable to cold acclimate. Other studies look at the possibility of crop improvement through more traditional approaches, by identifying *CBF/DREB1* from more freezing tolerant cultivars of the same species and transferring them into less freezing tolerant cultivars such as; the studies carried out in *Solanum tuberosum* cv. Umatilla (potato) (Pino et al., 2007), or *V. corymbosum* (Moore, 1993). Other research aims to transfer *CBF* genes from species such as *Arabidopsis* which can cold acclimate into chilling sensitive crops such as *Lycopersicon esculentum* (tomato) (Zhang et al., 2004) to improve chilling tolerance. As chilling sensitive plants are damaged by low positive temperatures and is therefore a separate form of cold stress to freezing damage, the same pathways such as the CBF/DREB1 pathway could be used to improve two different forms of stress tolerance in plants.

The work in this thesis introduces a new area of research, studying freezing tolerant plants could be used to further validate or disprove current theories on freezing tolerance, and has the potential to open up the possibility of identifying new genes or pathways of importance in cold acclimation and freezing tolerance. Studies on Arctic species to which freezing tolerance and effective cold acclimation is essential to survival would improve the field of cold acclimation and freezing tolerance research. Novel mechanisms or potential targets for crop improvement may be identified through comparative studies between Arctic plants and plants from warmer areas where freezing tolerance is less essential. For example comparisons of transcriptomics data obtained from Arctic populations of *V. myrtillus*, with data from a population found in warmer regions such as the south of France, or *Empetrum nigrum* populations which can be found in the Arctic and also in northern Asia (Bell & Tallis, 1973).Comparing results obtained from these populations under both cold and ambient conditions could potentially identify target genes or pathways of interest.

Carried out alongside comparisons of the whole genome sequences from each population as there may be small differences in sequence between genes from each population; results shown in this thesis suggest that small differences in amino acid sequence can have a large impact on the behaviour of the protein produced. Identified targets could then be further studied in a broader comparative study between large groups of plants from Arctic environments with plants from temperate regions. These comparative studies may highlight new pathways or gene targets alternatively; it may further highlight and emphasise the importance of pathways or genes that have already been identified, such as the CBF/DREB1 pathway. This could then progress into studying such targets in crop plants.

Further studies on arctic species focussing on cold acclimation and freezing tolerance could also be of importance in predicting the future effects of climate change on Arctic environments. Data collected after previous winter warming events and pest outbreaks (Bjerke, 2014) could be combined with more detailed information on how individual species survive and adapt to freezing; to more accurately model changes in overall species composition in response to climatic events such as altered snow depth, precipitation and winter warming events. This information could then be used to predict the wider impacts this will have on the sub-arctic environment such as changes in animal populations and carbon flux.

An outward looking perspective of the work presented in this thesis shows the importance of studying Arctic plants as a model system for further understanding cold acclimation and freezing tolerance in plants.

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Appendix A -Solutions and Media recipes

Nucleic acid extraction buffers

Edwards' extraction buffer
25mM EDTA
200mM Tris-HCl
250mM NaCl
0.5% (w/v) SDS

1x TE buffer
0.25M EDTA
1M Tris-HCl (pH 8.0)
of distilled water

CTAB (DNA) extraction buffer
2% CTAB (w/v)
1.42M NaCl
20mM EDTA
100mM Tris-HCl (pH 8.0)
2% PVP 40 (w/v) polyvinylpyrolidone)
5mM Ascorbic acid
4mM DIECA (diethylolithiocarbamic acid)

CTAB (RNA) extraction buffer
2% CTAB (w/v)
2% PVP 360 (w/v)
100mM Tris-HCl (pH 8.0)
25mM EDTA
2M NaCl
0.5g/litre spermidine
0.1% DEPC (v/v)

Lithium Chloride solution
10M lithium chloride
0.1% DEPC (v/v)

SSTE buffer
1M NaCl
0.5% SDS (v/v)
10mM Tris-HCl (pH 8.0)
1mM EDTA (pH 8.0)
0.1% DEPC (v/v)

Agarose Gel electrophoresis

0.5x TBE buffer
1mM EDTA
45mM Tris-Boric acid (pH 8.0)
Strep-tagged protein isolation

Basic extraction buffer
100mM Tris-HCl (pH 7.5)
100mM NaCl
1mM EDTA

Extraction Buffer
Just before use the following were added to basic extraction buffer:
10mmM ascorbic acid
10mM sodium hydroxide
10µg ml^{-1} DNAse 1
10µg ml^{-1} RNAse A
1ml / 30g of tissue plant protease inhibitor cocktail
1 tablet/ 10ml Phos-stop protease inhibitor cocktail
20mM sodium fluoride
20mM sodium orthovanadate
2mM PMSF
20mM Benzamidine

Desthiobiotin
2.5mM desthiobiotin made up in basic extraction buffer

HABA
1mM HABA (2-(4'-hydroxy-benzeneazo)-benzoic acid) made up in basic extraction buffer

GFP pull-downs
Extraction buffer
150mM Tris-HCl pH 7.5
150mM NaCl
10% glycerol
1% (v/v) Np-40
10mM EDTA
1mM sodium molybdate
1mM NaF
10mM DTT *
1% (w/v) PVPP *
1% (v/v) protease inhibitor cocktail (Sigma) *
0.5mM PMSF * *Add 30mins before use

Western blots

1.5% acrylamide resolving gel
4.6ml distilled water
10ml 30% acrylamide
5ml 1.5M Tris (pH 8.0)
0.2ml 10%SDS
0.2ml 10% ADS
8µl TEMED

Stacking gel

3.4ml distilled water
0.83ml acrylamide
0.63ml 1M Tris pH 6.8
0.05ml ADS
5µl TEMED
1 drop of bromophenol blue

10x PBS
80g NaCl
20g KCl
14g Na_2HPO_4
2.4g KH_2PO_4
Made up to 1 litre in distilled water
(Diluted to 1x before use)

10x Western running buffer
30.3g of Tris Base
144.2G Glycine
100ml 10%SDS
pH to 8.6 in 800ml of distilled water then top up to 1litre

Transfer buffer
3g Tris
14.4g Glycine
4ml 10% SDS
200ml methanol

10x TBS (Stock solution)
24.23g Tris
80.06g NaCl
pH to 7.6 with concentrated HCl then top up to 1l in distilled water

1x TBS
100ml of 10xTBS stock solution added to 900ml of distilled water

TBST
1ml of Tween 20 added to 999ml of 1xTBS

Silver staining

Fix solution
40% Methanol
10% Acetic acid

Sensitize solution
75ml Methanol
0.5g Sodium thiosulphate
17g Sodium acetate
Made up to 250ml total volume in milliQ water

Silver stain solution

75ml Methanol
0.5g Sodium thiosulphate
17g Sodium acetate
Made up to 250ml with milliQ water

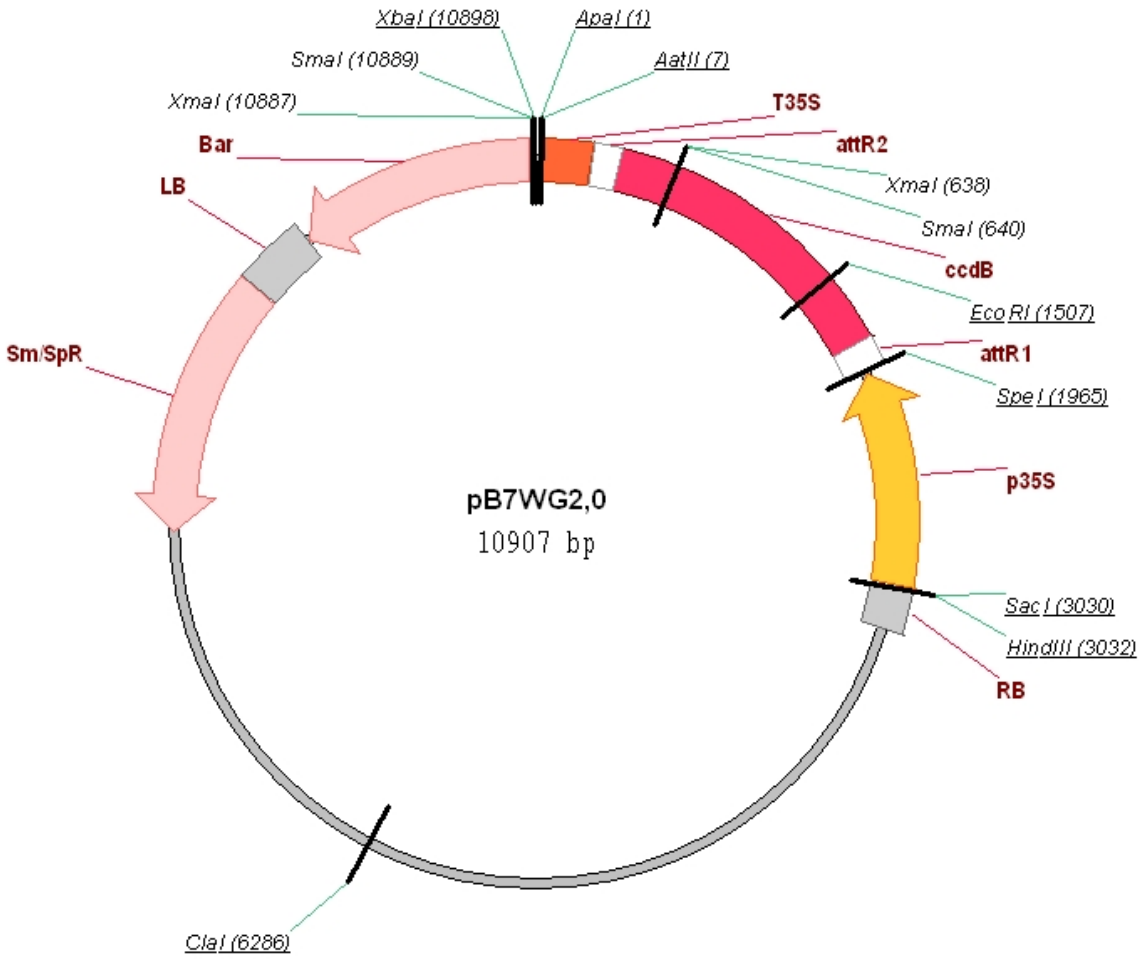
Silver stain solution
0.625g Silver nitrate
Made up to 250ml total volume in milliQ water

Developing solution
6.25g Sodium carbonate
Made up to 250ml total volume in milliQ water
100µl Formaldehyde solution added just before use

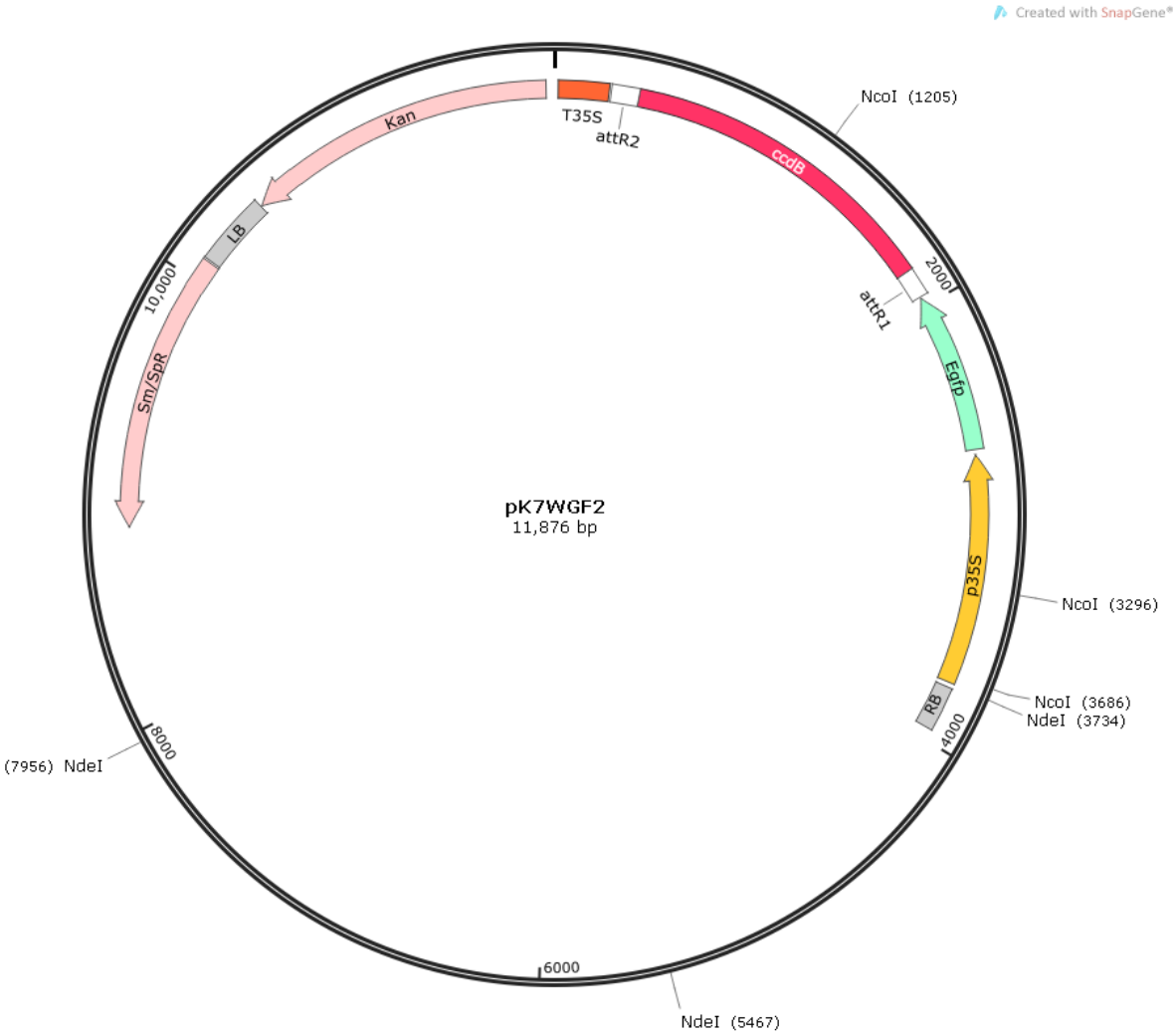
Stop solution
3.65g EDTA
Made up to 250ml total volume in milliQ water

Appendix B -Ghent vector maps

A. Vector map of plasmid pB7WG2



B. Vector map of plasmid pK7WGF2



Appendix C –List of primers

A. Primers used for PCR and cloning

	Primer	Sequence	Species
	CBF-VI-F	CACCGAAGAGTTAGATGTGCGCAGC	*Vaccinium*
	CBF-VI-R	ATCTAACTCCACAAGAGACC	*Vaccinium*
	Myrtillus CBF-F	CACCATGGAATATTACTCAAGTCC	*V. myrtillus*
	Uli-vitis- CBF-F	CACCATGGAATATAACTCTAGTCC	*V. uliginosum, V. vitis-idaea*
	myrtillus CACC-MEY-CBF-F	CACCATGGAATATTACTCAAGTCC	*V. myrtillus*
	Uli-Vitis-MEY-CBF-CACC-F	CACCATGGAATATAACTCTAGTCC	*V. uliginosum, V. vitis-idaea*
	CACC-CBF-VI-F	CACCGAAGAGTTAGATGTGCGCAGC	*Vaccinium*
	CBF1-F	TCGATAGTCGTTTCCATTTTTG	*Arabidopsis*
	CBF1-R	TTTTCCACTCGTTTCTACAACA	*Arabidopsis*
	CBF2-F	TGATGTCGAGGGAGATGATG	*Arabidopsis*
	CBF2-R	AAAAAGCTATAATACCAAAAATGGAAA	*Arabidopsis*
	CBF3-F	GAGAAACTATTATTTCAGCAAACCA	*Arabidopsis*
	CBF3-R	TCTGTTTCAAGAAACTGGATAAGG	*Arabidopsis*
	GFP-GW-F	CGCGCTGCAGATAACAATGGTGAGCA AGGGCGAGGA	*Arabidopsis*
	GFP-GW-R2	CGCGCCCGGGTTACTTGTACAGCTCGT CCA	*Arabidopsis*
	VviEST-F	ATGGCTGCTCGGGCTCAGGATGTAG	*Vaccinium*
	VviEST-R	CTCCACAACGAGACCTCAACATCACTTC	*Vaccinium*
	HMBF	AGTTAGACATTTGCCCATACAC	*Vaccinium*
	HMBR	CTAACTCCACAACGAGACCTCAAC	*Vaccinium*
short sequences	VESK1-F	ATGCAGCC ACCCCGCCGG	*Vaccinium*
	VESK2-F	TGTTATCCACCGTAACCCTC ACA	*Vaccinium*
	VESK1-R	GGCAAAGAGCAATCTCCTTG	*Vaccinium*
	VESK2-R	GAACCTGCAACAATCTTGC	*Vaccinium*
	VICE1-F	AGCTCGGAAG ATATCCAACG	*Vaccinium*
	VICE2-F	CCTTC TCTCAACCTC ACTTCGAC	*Vaccinium*
	VICE1-R	CCTTCCCTCCCTTGCCGCAT	*Vaccinium*
	VICE2-R	CTCCAGAAGATCAATGACCT	*Vaccinium*
	VHOS11-F	CAATCACGCA ATTCCCATTC	*Vaccinium*
	VHOS12-F	ACTTGATTGC TGCCACGTCA C	*Vaccinium*
	VHOS11-R	CGTAATCCGAATGTCTCGGACC	*Vaccinium*
	VHOS12-R	CCAATCCGAACCGCGAGTCAC	*Vaccinium*
	VMYB1-F	ATTCAG GTAGACTGGC GGCTC	*Vaccinium*
	VMYB2-F	GACG AGAAGGTTGT ACC	*Vaccinium*
	VMYB1-R	TCGGACCGATAACGAGATC	*Vaccinium*

	VMYB2-R	CGCTG CGATTGCAGA CCATCT	*Vaccinium*
	VSIZ1-F	AGAT GCGGAATTGT GAGGAAG	*Vaccinium*
	VSIZ2-F	CAGAGAT TGAGGTGAAGCCTG	*Vaccinium*
	VSIZ1-R	CAAGGCCTCTGATGCATCTG	*Vaccinium*
	VSIZ2-R	GCAGATCTAATAGCAGTCT	*Vaccinium*
	VHOS91-F	AGAGAGT GCTGCAATGA GAG	*Vaccinium*
	VHOS92-F	AGAGGCAAAT TCCAACTTCTTC	*Vaccinium*
	VHOS91-R	TCCCATTGTGTAATGCATG	*Vaccinium*
	VHOS92-R	CATGGAGATTGCTAATTCTTC	*Vaccinium*
	VMYB2-R	CGCTG CGATTGCAGA CCATCT	*Vaccinium*
	VSIZ1-F	AGAT GCGGAATTGT GAGGAAG	*Vaccinium*
	VSIZ2-F	CAGAGAT TGAGGTGAAGCCTG	*Vaccinium*
	VSIZ1-R	CAAGGCCTCTGATGCATCTG	*Vaccinium*
	VSIZ2-R	GCAGATCTAATAGCAGTCT	*Vaccinium*
	VHOS91-F	AGAGAGT GCTGCAATGA GAG	*Vaccinium*
	VHOS92-F	AGAGGCAAAT TCCAACTTCTTC	*Vaccinium*
	VHOS91-R	TCCCATTGTGTAATGCATG	*Vaccinium*
	VHOS92-R	CATGGAGATTGCTAATTCTTC	*Vaccinium*

B. RACE PCR primers

Number	Primer name	Sequence	Species
1	RC-VICE1-F	CGTTGGATATCTTCCGAGCT	All 3 were identical
2	RC-VICE-2-F	GGTCTCTTGCTGTCAACTATGC	All 3 were identical
3	RC-VICE-3-F	CATTGGTTATGCTGCAACAATCC	All 3 were identical
4	RC-VICE-3-R	CCTGAGAATATCGTAGGCAGC	All 3 were identical
5	RC-VICE-2-R	GAAGATGTACCCTTGCAGGCTG	All 3 were identical
6	RC-VICE1-R	ATGCGGCAAGGGAGGGAAGG	All 3 were identical
7	RC-VMYB1-F	GAGCCGCCAGTCTACCTGAAT	All 3 were identical
8	M-RC-MYB-2-F	GCAAGAAGCCTGCGGATAG	*V. myrtillus*
9	M-RC-MYB-3-F	CTCTGAATTCCCGGATTC	*V. myrtillus*
10	M-RC-MYB-3-R	GCGGATGATAATCAAGTGGATG	*V. myrtillus*
11	M-RC-MYB-2-R	CCAACGACGAGTTAGGATTTC	*V. myrtillus*
12	RC-VMYB1-R	GATCTCGTTATCGGTCCGA	All 3 were identical
13	IU-RC-MYB-2-F	CGACGAATACTAGTACTACTAC	*V. uliginosum, V. vitis-idaea*
14	IU-RC-MYB-3-F	GCGGATGATGATCAAGTGGATG	*V. uliginosum, V. vitis-idaea*
15	IU-RC-MYB-3-R	GAAACGGGTTCGTACCCTGGACT	*V. uliginosum, V. vitis-idaea*
16	IU-RC-MYB-2-R	GAAGCATTGGAATCGGATTTGC	*V. uliginosum, V. vitis-idaea*
17	RC-VHOS92-F	GAAGAAGTTGGAATTTGCCTCT	All 3 were identical
18	RC-HOS9-3-F	GCTGATAATGTCTGCCTTATGG	All 3 were identical
19	RC-HOS9-4-F	CCTTAATTGACTCTTGATGTAGTC	All 3 were identical
20	RC-HOS9-4-R	GGTGAAAGGAAGAGAAAATCG	All 3 were identical
21	MU-RC-HOS9-3-R	CATGCATTACACAATGGG	*V. myrtillus, V. uliginosum*
22	I-RC-HOS9-3-R	CTTGATTTCCCCAAGGGG	*V. vitis-idaea*
23	RC-VHOS92-R	GAAGAATTAGCAATCTCCATG	All 3 were identical
24	M-RC-VESK-3-F	CATTGTGAGGGTTACGGGGGGTAA	*V. myrtillus*
25	Vi-RC-VESK-3-F	CGTTGAGGAGGTGGGGGGAGTAAC	*V. vitis-idaea*
26	U-RC-VESK-3-F	CATTGGGAGGGTTACGGGGGATCA	*V. uliginosum*
27	M-RC-VESK-4-F	ACCACTACAAACAGAGAGAGG	*V. myrtillus*
28	Vi-RC-VESK-4-F	ACCACTACCAACAGGAGGGTG	*V. vitis-idaea*
29	U-RC-VESK-4-F	ACCACTACAAATAGAGAGAGG	*V. uliginosum*
30	MU-RC-VESK-5-F	TCTGCTATTGACTTCACGTCCTCG	*V. myrtillus, V. uliginosum*
31	Vi-RC-VESK-5-F	GCTGTTGATGACTCCACGTCGTCG	*V. vitis-idaea*
32	M-RC-VESK-5-R	CCACGCACCCGATTTATAGA	*V. myrtillus*

33	Vi-RC-VESK-5-R	CACCGCACCCCCCGAACAGCG	*V. vitis-idaea*
34	U-RC-VESK-5-R	CCACGCACCCGATTTACAGA	*V. uliginosum*
35	MU-RC-VESK-4-R	GTGGCAGCCCAGAGATTGC	*V. myrtillus, V. uliginosum*
36	Vi-RC-VESK-4-R	ATGGCAGCCCACCCGATGG	*V. vitis-idaea*
37	M-RC-VESK-3-R	CACACACACAATATAGGAATA	*V. myrtillus*
38	Vi-RC-VESK-3-R	GATGGACGAACAAAGCATGTT	*V. vitis-idaea*
39	U-RC-VESK-3-R	TCGACAACATGATTGGATGTA	*V. uliginosum*

C. QPCR primers

Primer	Sequence	Species
VviDreb1-R	AAGAAGGCGAGGGAGAGAGT	*Vaccinium*
Vvi-Dreb1 F	GGGAATGGGAGTGAGGTTTT	*Vaccinium*
KIN2 F	CTGGCAAAGCTGAGGAGAAG	*Arabidopsis*
KIN2 R	ACTGCCGCATCCGATATACT	*Arabidopsis*
COR15a F	CGTTGATCTACGCCGCTAAAG	*Arabidopsis*
COR15a R	CTACACCATCTGCTAATGCC	*Arabidopsis*
COR414 F	GGGAGAGTATGGTGTATGGGCA	*Arabidopsis*
COR414 R	TGATATGGCGCCACAATCA	*Arabidopsis*
GOLS3 F	CAAAGTTGTCCCTCCCACAC	*Arabidopsis*
GOLS3 R	GAGCATGGCCAAGACAAGAT	*Arabidopsis*
PEX4 F	TCATAGCATTGATGGCTCATCCT	*Arabidopsis*
PEX4 R	ACCCTCTCACATCACCAGATCTTAG	*Arabidopsis*
LTI78-F	GCACCCAGAAGAAGTTGAACA	*Arabidopsis*
LTI78-R	TCATGCTCATTGCTTTGTCC	*Arabidopsis*
Tubulin F	AGGAAATGTTTAGGCGTGTGAGC	*Vaccinium*
Tubulin R	AGTGAACTCCATCTCGTCCATACC	*Vaccinium*
Vaccinium DREB1 RT-R	AAGAAGGCGAGGGAGAGAGT	*Vaccinium*
Vaccinium DREB1 RT-F	GGGAATGGGAGTGAGGTTTT	*Vaccinium*
RT_V_ESK1_F	CTCTGGGCTGCCCACCTCC	*Vaccinium*
RT_V_ESK1_R	GGTGGAATTGCCGCCGGAAG	*Vaccinium*
RT_V_MYB15_F	GTGCCACCTATGATTGGGC	*Vaccinium*
RT_V_MYB15_R	GGATGGTCACATGACCACCC	*Vaccinium*
RT_V_ICE2_F	GGTTATGCTGCAACAATCC	*Vaccinium*
RT_V_ICE2_R	GAAGCAGCTGATAACAGCTTG	*Vaccinium*
RT_MU_SIZ1_F	GGTGGAACCACCAGCATCAG	*Vaccinium*
RT_MU_SIZ1_R	CAGACCACAATTCTGCACTG	*Vaccinium*
RT_I_SIZ1_F	GCAGAATTCGCCCT TAGATGCGG	*Vaccinium*
RT_I_SIZ1_R	TGCCACCGCCCTAGATCTCC	*Vaccinium*

Appendix D- GenBank submissions

Gen bank submission pages for *CBF/DREB1* sequences from the three *Vaccinium* species.

Vaccinium myrtillus DREB1 gene, complete cds

GenBank: JN254610.1

FASTA Graphics

Go to:

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13-MAR-2013
DEFINITION  Vaccinium myrtillus DREB1 gene, complete cds.
ACCESSION   JN254610
VERSION     JN254610.1  GI:351589912
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SOURCE      Vaccinium myrtillus
  ORGANISM  Vaccinium myrtillus
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Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons;
Gunneridae;
            Pentapetalae; asterids; Ericales; Ericaceae;
Vaccinioideae;
            Vaccinieae; Vaccinium.
REFERENCE   1  (bases 1 to 737)
  AUTHORS   Oakenfull,R.J., Baxter,R. and Knight,M.R.
  TITLE     A C-repeat binding factor transcriptional activator
(CBF/DREB1)
            from European bilberry (Vaccinium myrtillus) induces
freezing
            tolerance when expressed in Arabidopsis thaliana
  JOURNAL   PLoS ONE 8 (1), E54119 (2013)
   PUBMED   23349799
  REMARK    Publication Status: Online-Only
REFERENCE   2  (bases 1 to 737)
  AUTHORS   Oakenfull,R.J., Knight,M.R. and Baxter,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-JUL-2011) Biological and Biomedical
Sciences, Durham
            University, South Road, Durham DH1 3LE, UK
FEATURES             Location/Qualifiers


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Vaccinium uliginosum DREB1 (DREB1) gene, complete cds

GenBank: JN866911.1

FASTA Graphics

Go to:

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13-MAR-2013
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Gunneridae;
            Pentapetalae; asterids; Ericales; Ericaceae;
Vaccinioideae;
            Vaccinieae; Vaccinium.
REFERENCE   1  (bases 1 to 737)
  AUTHORS   Oakenfull,R.J., Baxter,R. and Knight,M.R.
  TITLE     A C-repeat binding factor transcriptional activator
(CBF/DREB1)
            from European bilberry (Vaccinium myrtillus) induces
freezing
            tolerance when expressed in Arabidopsis thaliana
  JOURNAL   PLoS ONE 8 (1), E54119 (2013)
   PUBMED   23349799
  REMARK    Publication Status: Online-Only
REFERENCE   2  (bases 1 to 737)
  AUTHORS   Oakenfull,R.J., Knight,M.R. and Baxter,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-OCT-2011) Biological and Biomedical
Sciences, Durham
            University, South Road, Durham DH1 3LE, UK
FEATURES             Location/Qualifiers


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Vaccinium vitis-idaea DREB1 (DREB1) gene, complete cds

GenBank: JN866912.1

FASTA Graphics

Go to:

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13-MAR-2013
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VERSION     JN866912.1  GI:379061446
KEYWORDS    .
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  ORGANISM  Vaccinium vitis-idaea
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Gunneridae;
            Pentapetalae; asterids; Ericales; Ericaceae;
Vaccinioideae;
            Vaccinieae; Vaccinium.
REFERENCE   1  (bases 1 to 736)
  AUTHORS   Oakenfull,R.J., Baxter,R. and Knight,M.R.
  TITLE     A C-repeat binding factor transcriptional activator
(CBF/DREB1)
            from European bilberry (Vaccinium myrtillus) induces
freezing
            tolerance when expressed in Arabidopsis thaliana
  JOURNAL   PLoS ONE 8 (1), E54119 (2013)
   PUBMED   23349799
  REMARK    Publication Status: Online-Only
REFERENCE   2  (bases 1 to 736)
  AUTHORS   Oakenfull,R.J., Knight,M.R. and Baxter,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-OCT-2011) Biological and Biomedical
Sciences, Durham
            University, South Road, Durham DH1 3LE, UK
FEATURES             Location/Qualifiers


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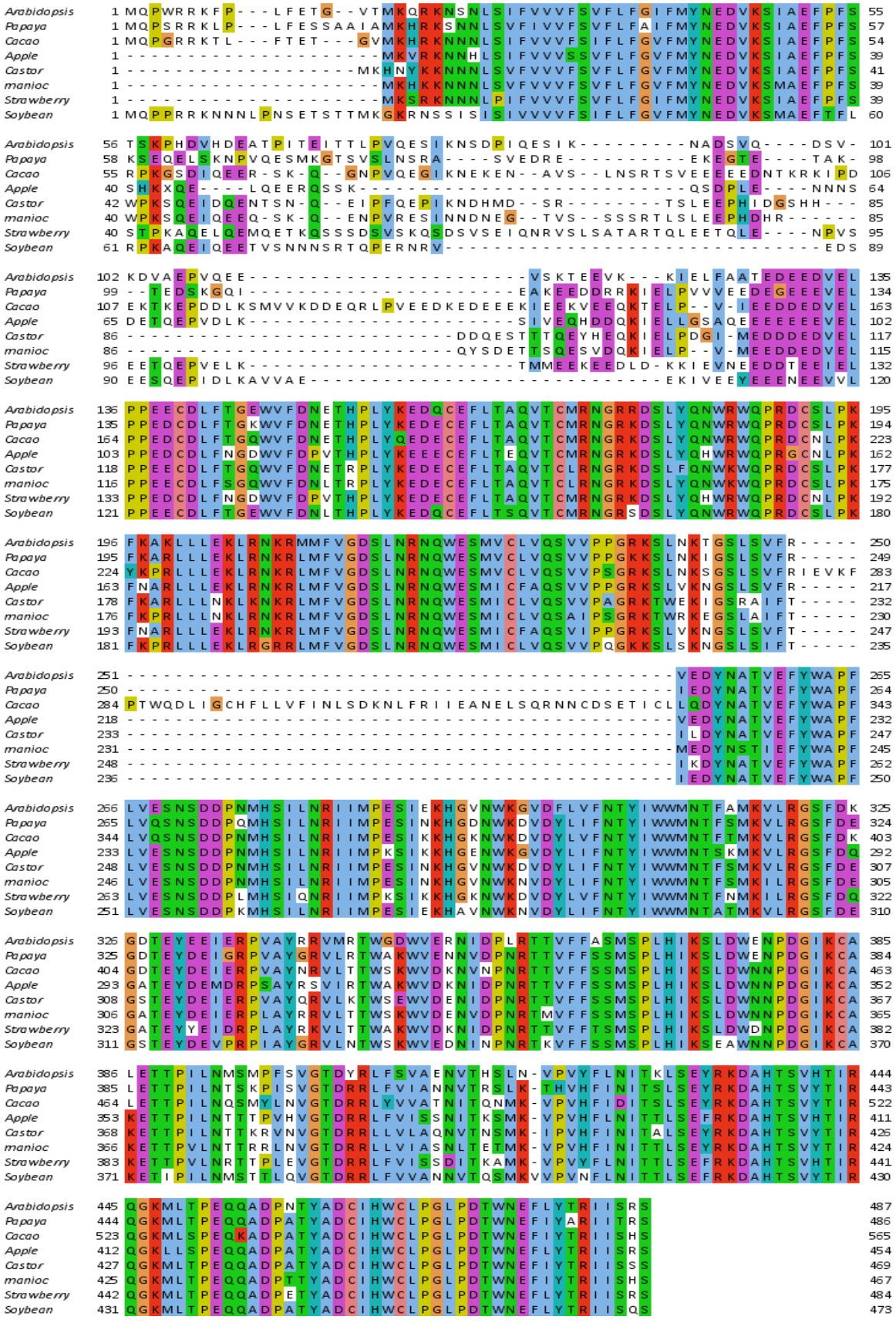
Appendix E –Clustal/Jalview line ups of *ESK1*



Appendix E –Theoretical digests for mass spectrometry

A. Protein sequences

e-GFP (from Ghent vector)
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V. myrtillus
MEYYSSPHSGAFPIMSNSGSKSPSLEYVEGSTSRGSPQSDEELMITLASSQPKKRAGRKKFKETRHPIYRGVR
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V. uliginosum
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WDDVGSDVEVSLWS"

V vitis-idaea
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B. –Theoretical digests

eGFP ions doubly charged iodoacetamide

1283.6502	217-239	0			DHMVLLEFVTAAGITLGMDELYK
1219.1341	5-27	0			GEELFTGVVPILVELDGDVNGHK
1189.6325	54-74	0	Cys_CAM: 71	1218.1432	LPVPWPTLVTTLTYGVQCFSR
987.4567	142-157	0			LEYNYNSHNVYIMADK
752.3335	28-42	0			FSVSGEGEGDATYGK
633.7928	87-97	0			SAMPEGYVQER
525.7643	115-123	0			FEGDTLVNR
491.7512	133-141	0			EDGNILGHK
411.2007	81-86	0			QHDFFK
395.6812	75-80	0			YPDHMK
385.1993	47-53	0	Cys_CAM: 49	413.7100	FICTTGK
356.1508	103-108	0			DDGNYK
328.1943	98-102	0			TIFFK
301.6426	211-215	0			DPNEK

V. myrtillus

1139.0012	1-21	0			MEYYSSPHSGAFPIMSNSGSK
1009.4909	35-53	0			GSPQSDEELMITLASSQPK
871.8937	117-132	0	Cys_CAM: 123	900.4045	GESSTACLNFADSVWR
821.3675	210-224	0			FSWDDVGSDVEVSLS
790.4005	93-106	0			LWLGTYPTAEMAAR
706.3386	22-34	0			SPSLEYVEGSTSR
533.7529	147-156	0			AAAEAAEMFR
518.8111	107-116	0			AHDVAALVLK
403.2049	80-85	0	Cys_CAM: 82	431.7156	WVCELR
377.6957	140-145	0			EAEHIR
370.2392	133-139	0			LPVPVSK
343.1926	66-70	0			HPIYR

V. uliginosum

1077.4814	1-21	0			MEYNSSPHSGAFPIGSNSGSK
914.4072	210-225	0			FSWDDVGSDVEVSLWS
886.8990	117-132	0	Cys_CAM: 123	915.4098	SESSTACLNFADSVWR
824.9085	39-53	0			SDEELMITLASSQPK
790.4005	93-106	0			LWLGTYPTAEMAAR
706.3386	22-34	0			SPSLEYVEGSTSR
504.7954	107-116	0			AHDVAALALK
403.2049	80-85	0	Cys_CAM: 82	431.7156	WVCELR
370.2392	133-139	0			LPVPVSK
343.1926	66-70	0			HPIYR
341.6852	140-145	0			EAGHIR

V. vitis-idaea

1218.0992	116-138	0	Cys_CAM: 122	1246.6099	GESSTACLNFADSVWQLPVP VSK
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914.4072	209-224	0			FSWDDVGSDVEVSLWS
824.9085	38-52	0			SDEELMITLASSQPK
790.4005	92-105	0			LWLGTYPTAEMAAR
714.3543	21-33	0			SISLEYVEGSTSR
533.7529	146-155	0			AAAEAAEMFR
504.7954	106-115	0			AHDVAALALK

403.2049	79-84	0	Cys_CAM: 81	431.7156	WVCELR
377.6957	139-144	0			EAEHIR
343.1926	65-69	0			HPIYR

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