Responses to
Low Temperature Stress in
Phaseolus Species

A Thesis Submitted to

The College of Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

in the Department of Plant Sciences

University of Saskatchewan

Saskatoon

By

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Abstract

Expansion of common bean (*Phaseolus vulgaris* L.) crops in the northern Great Planes has been hampered due to the lack of cultivars demonstrating sufficient vitality under low temperature conditions. *Phaseolus angustissimus* L., a wild bean species, has been previously shown to possess the ability to survive low temperatures in field trials. Freezing tolerance experiments under controlled conditions resulted in *P. angustissimus* demonstrating a greater capacity for freezing tolerance than *P. vulgaris*, as all *P. vulgaris* plants studied were dead at -2.5^{o}C while most *P. angustissimus* plants treated to the same conditions survived. Exposure to chilling temperatures over five days resulted in stunted growth in both species, but the cultivated bean suffered more compared to the wild bean, as noted by a marked loss in tissue water content over the first three days of chilling. Interspecific macroarray hybridizations of a cDNA library from cold acclimated *Medicago sativa* L. using cDNAs derived from non-chilled and three-day chilled *P. vulgaris* and *P. angustissimus* plants showed that *P. vulgaris* showed more changes in gene expression after three days of chilling. Also, *P. vulgaris* showed a general trend towards down-regulation of the transcripts sampled on the third day of chilling compared to *P. angustissimus*. RT-PCR experiments were conducted using cDNAs from plant tissues exposed to various durations of chilling to confirm the results from the macroarray experiment. These time-course RT-PCR experiments revealed expression patterns across various chilling durations in genes identified from the macroarray. Data from these experiments suggest that *P. vulgaris* and *P. angustissimus* seedlings respond differently to low temperature exposure, and that some of the changes in *P. angustissimus* transcripts monitored here may be useful for

researchers in better understanding how *Phaseolus* species can respond better to chilling temperatures.

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Acknowledgements

I would like to thank my co-supervisors Drs. Kirstin Bett and Bert Vandenberg for allowing me to pursue research in their laboratories under their guidance. Additionally, I would like to thank members of the Bett lab, past and present, for their assistance in my various laboratory-related endeavors.

I would also like to thank members of my advisory committee; Dr. Isobel Parkin for her assistance technically and conceptually with regards to macroarrays, Dr. Hugo Cota-Sanchez for stressing the importance for evolutionary conceptions, Dr. Gordon Gray for his assistance in nearly all aspects of experimental design in addition to other issues of great importance, and Dr. Mary O'Connell for being an insightful external examiner. I give thanks to Dr. Serge Laberge and his research team (Rejean and David) in Ste-Foy, Quebec for access to their *Medicago sativa* cDNA library, genomics equipment, expertise, and friendship during my collaboration and beyond.

I am deeply grateful to my parents, Fay and Rob, for their seemingly limitless support for my personal and academic goals. I am greatly indebted to Nadine Poulton for her never-ending support and assistance in all aspects of my life, and being there for me every step of the way.

The funding for this research was provided by the Saskatchewan Pulse Growers, NSERC, Paulden F. and Dorathea I. Knowles Postgraduate Scholarship, and the University of Saskatchewan's University Graduate Fellowship.

"*When you cut into the present the future leaks out.*" -- William S. Burroughs

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LIST OF ABBREVIATIONS

A	absorbance wavelength
AAFC	Agriculture and Agri-Food Canada
ABA	abscisic acid
ABI	abscisic acid insensitive
AFP	antifreeze protein
AP	APETALA
ARF	Auxin responsive factor
ATP	adenosine triphosphate
bHLH	beta helix-loop-helix
BLAST	basic local alignment search tool
CAP	cold acclimation protein
cas	cold acclimation-specific
CBF	cytosine repeat binding factor
cDNA	complimentary DNA
CIP	constitutive photomorphogenic (COP) interactive protein
CO_2	carbon dioxide
CoA	coenzyme A
COR	cold regulated
CV	coefficient of variance
cv.	cultivar, cultivated variety
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleic acid

DRE	dehydration responsive element
DREBP	DRE binding protein
EDTA	ethylenediamine disodium tetra-acetate
EL	electrolyte leakage
EREBP	ethylene response element binding protein
FAD	fatty acid desaturase
GABA	gamma-aminobutyric acid A
HCl	hydrogen chloride
HSP	heat shock protein
ICA	International Center for Agricultural
ICE	inducer of CBF expression
kDa	kilo Dalton
LEA	late embryogenic abundant
LT	lethal temperature
m	meters
min	minute
mL	millilitre
mM	milli Molar
M-MLV	moloney murine leukemia virus
mRNA	messenger RNA
n	population number
NaOH	sodium hydroxide
nM	nano Molar

ºC	degrees Celsius
PCR	polymerase chain reaction
PDC	pyruvate decarboxylase
PPFD	photosynthetic photon flux density
Py D	pyruvate decarboxylase isozyme 1
RAV	related to ABI3/VP1
RH	RNA helicase
RNA	ribonucleic acid
ROS	reactive oxygen species
RRM	RNA recognition motif
RT	reverse transcriptase
s	second
SDS	sodium dodecyl sulfate
SSC	salt sodium citrate
TRIS	trishydroxymethylaminomethane, 2-amino-2-hydroxymethyl-1,3-propanediol
UV	ultraviolet
VP	*Viviparous*
vs.	versus
µ	micro
µL	micro litre
µM	micro Molar

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is the world's largest pulse crop in terms of production and trade volume, exceeding that of lentil, chickpea, and field pea (Saskatchewan Pulse Growers, 2000). Production of common bean in Canada is concentrated in Manitoba and Ontario with additional acreage in pockets of Saskatchewan and Alberta. In 2005, the total area seeded for common bean was 163 000 ha in Canada, producing 220 000 tonnes (Market Analysis Division, Agriculture and Agri-Food Canada, 2006). In 2000, Saskatchewan farmers grow 3238 ha of common bean under irrigation (Saskatchewan Pulse Growers, 2000). Low acreage of common bean production in Saskatchewan can be partly attributed an insufficient number of frost-free days, particularly in the Dark Brown and Black soil zones which are the best-suited for common bean growth. Researchers at the University of Saskatchewan, Saskatoon, are selecting for early maturing common bean varieties that are better adapted to this short-season environment. Additionally, selection of germplasm displaying superior ability to emerge at low seedbed temperatures as well as surviving spring frosts are also major objectives of the common bean breeding program.

Normal growth of common bean is impaired at temperatures below 15 ºC. Seedbed temperatures below 15 ºC also results in inhibition of germination and emergence of common bean seedlings (Dickson and Boettger, 1984). Chilling

injury can be somewhat offset by increasing the vigor of the seedlings in cool conditions, either by fungicide application or by maintaining a seed moisture content at or above 12% (Roos and Manalo, 1976). Studies have been conducted with wild relatives of common bean to identify genotypic differences with regard to germination and emergence (Kooistra, 1971; Dickson and Boettger, 1984; Zaiter et al., 1994). The reduced germination and emergence of common bean seeds in seedbeds at suboptimal temperatures can be ameliorated upon onset of warmer temperatures (Balasubramanian et al., 2004). Thus, chilling temperatures 'set back' plant growth, resulting in delayed maturity and an increased risk of exposure to lethal early fall frosts. Balasubramanian et al. (2004) studied the emergence of various lines of *P. vulgaris* under cool (<15 ^{o}C) seedbed temperatures and concluded that while such temperatures delayed emergence, dry beans seedlings suffered little permanent damage. Development of low temperature tolerant common bean varieties would enable farmers to plant earlier in the spring in order to achieve maturity prior to fall frosts.

Radiation frost on clear nights results in mortality in common bean seedlings during the growing season. Exposing common bean plants to extrinsic ice nucleators is lethal in controlled environments as well as in the field (Ashworth et al., 1985; Balasubramanian, 2002). However, common bean is capable of supercooling to -7 ^{o}C in the absence of ice nucleators (Balasubramanian, 2002). Even in the absence of freezing temperatures, chilling sensitive plants display characteristic responses to chilling temperatures. Additionally, some temperate

species are known to increase their freezing tolerance after exposure to low, non-freezing temperatures, a process termed 'cold acclimation' (Thomashow, 1999).

Transcriptional profiling of many plant species has resulted in the identification of a variety of low temperature regulated genes, some of which may be involved in cold acclimation (Shinozaki et al., 2000). While studies of low temperature induced gene expression have been undertaken in a variety of species, there is little information with regard to alterations in gene expression in bean species. Knowledge of changes in gene expression in response to chilling would enable researchers to pursue new methods of increasing freezing and chilling tolerance in bean species, with the goal of developing common bean cultivars better suited for cultivation in the northern prairies. However, given the paucity of genomic resources to carry out such research in *Phaseolus*, using legumes that have a greater amount of such resources, such as *Medicago sativa* L., could be used to profile transcription in chilled beans.

Breeders commonly use wild relatives of their crop as sources of desirable traits to improve crops. A wild relative of common bean, *Phaseolus angustissimus* L., has demonstrated a greater ability to survive subzero temperatures in field conditions compared to *P. vulgaris* (Balasubramanian et al., 2004). Obtaining an understanding of how *P. angustissimus* survives low temperatures better than *P. vulgaris* is an important step in the development of low temperature tolerant but edible *P. vulgaris* lines. Understanding why *P. angustissimus* survives better under low temperature conditions can enable researchers to screen for similar patterns of gene expression in developing a low temperature tolerant cultivar of common bean.

Suboptimal temperatures early in the growing season delay maturity of common bean varieties, increasing the likelihood of experiencing a lethal frost at the beginning and the end of the growth season. The general hypothesis for this work was that the wild bean species, *P. angustissimus*, survives better under low temperature conditions than the cultivated bean species, *P. vulgaris*. In order to evaluate this hypothesis, the objectives of this research were to:

1. Determine the level of freezing tolerance after a period of low temperature exposure in *P. vulgaris* and *P. angustissimus*.
2. Document some physiological responses, such as changes in height, water content, and chlorophyll content, to chilling temperatures in *P. vulgaris* and *P. angustissimus*.
3. Profile transcription of a number of chilling related genes in *P. vulgaris* and *P. angustissimus* before and after a chilling event to identify and characterize selected cold responsive genes.

2. Literature review

2.1 Common bean

Common bean (*Phaseolus vulgaris* L.), also called field bean or dry bean, is an ancient crop native to South and Central America. Common bean is primarily grown in areas of eastern Africa, eastern Asia, southeastern Europe, and the Americas, with Brazil being the world's leader in common bean production and consumption (Food and Agriculture Organization, 2002). Common bean is the world's number one pulse crop in terms of production, as 16-17.5 million tonnes are produced worldwide over 25 million hectares (Food and Agriculture Organization, 2002). The most widely traded pulse crop in the world is common bean (Food and Agriculture Organization, 2002). Brazil, Mexico, the United Kingdom, and Japan are considered to be major importers of common bean, while major common bean exporters include China, USA, Canada, and Argentina (Saskatchewan Pulse Growers, 2003).

Common beans are a great source of human nutrition, as they are high in protein, fiber, complex carbohydrates, minerals and vitamins (International Center for Tropical Agriculture (CIAT), 2001). Half the daily requirement of folic acid, a B-vitamin that is important for expecting mothers, can be found in a cup of common bean. Common bean is also an important source of dietary iron, magnesium, and copper.

Saskatchewan production of common bean is usually centered in the moist brown and black soil zones, and in the irrigated regions of Lake Diefenbaker

(Saskatchewan Pulse Growers, 2000). Since common bean is susceptible to major damage due to frost, one of the limitations of common bean production in Saskatchewan is the episodic freezing events that can occur in late spring and early fall (Balasubramanian, 2002). Typically, adapted common bean varieties require 90-120 frost free days to reach maturity (Saskatchewan Pulse Growers, 2000). Saskatchewan common bean growers are advised to plant the earliest maturing varieties in order to limit the risk of exposing common bean crops to late season frosts.

In attempts to identify suitable common bean relatives that may be a source of gene transfer via breeding experiments, Balasubramanian (2002) assayed levels of frost tolerance of a number of *P. vulgaris* accessions in addition to some species of the tertiary gene pool. Field results from the 2001 and 2002 Saskatoon growing seasons showed that *Phaseolus angustissimus* L., a member of the tertiary gene pool of *P. vulgaris*, had the greatest ability to survive radiation frosts when temperatures dropped to -7^{o}C. *P. angustissimus* originates from the southern United States in the high altitude regions, ranging from 1500-2000 m above sea level (Buhrow, 2007). Balasubramanian (2002) concluded that avoiding freezing damage via supercooling (see below) was a primary mechanism enabling *P. angustissimus* to survive sub-zero temperatures, Consequently, understanding the physiological and molecular mechanisms *P. angustissimus* employs to better survive freezing temperatures would assist researchers in transferring and/or improving low temperature tolerance in the cultivated common bean.

2.2 Low temperature stress

Temperature and hours of daily sunlight are two of the most important factors affecting crop distribution on Earth, followed by moisture and soil profile at the regional level (Blum, 1988). Plant growth and development patterns distinctly follow temperature response profiles, with optimum temperature ranges for biogenetic processes to take place. Temperatures outside of the optimum ranges can be considered as imposing a stress on the plant.

Optimal growth temperature is a fundamental principle in plant biology. Since plants are sessile and cannot control environmental temperature, they have developed two major strategies for surviving suboptimal temperatures: they either avoid the stress, or tolerate it. With the exception of a few plants that produce heat by metabolism (Knutson, 1974), plants must avoid seasonal extremes in temperature by other mechanisms than those employed by animals. Although plants species have developed strategies to tolerate the range of temperatures common to their natural habitat, injury may occur when the same plant species is grown in a new environment, or when temperatures in their native environment become extreme. Modern agriculture and consumer preferences have driven lucrative crop plants to be grown in regions where it may be economically worth the risk to grow them, but where they may be poorly adapted.

Plants of tropical and subtropical origin often suffer from chilling injury at non-freezing temperatures below 10ºC. Two forms of chilling stress have commercial significance. The first concerns plant growth and development in the early period of the growing season when temperatures are low. Crops that originated from tropical and/or

subtropical regions suffer injury when above-ground temperatures fall below a critical threshold. This injury can have a massive impact on the value of a particular crop, as demonstrated by the estimated $60 million loss to cotton producers in 1980 (Wilson, 1984). That year, unseasonably low temperatures damaged cotton seedlings, resulting in widespread crop failure. Although low temperatures impact the growth and development of chilling susceptible crops, only in extreme instances does it cause crop failure. However, the threat of prolonged periods of low temperatures restricts susceptible crops to specific geographical regions that have historically shown low probabilities of damaging temperatures.

Chilling is most detrimental on the flowers of plants. Pollen development and flower induction are particularly affected by chilling temperatures. In some chilling sensitive species, male sterility can result from prolonged exposure to chilling temperatures.

The second critical impact of chilling on agriculture is post-harvest storage of vegetation and seeds prior to market. The quality of fruits and vegetables after harvest is often maintained by refrigeration after harvest, but some commodities cannot be stored at low temperatures because of ripening and spoilage considerations. One notable example of post-harvest ripening is banana (*Musa* spp.), but storage considerations are also critical for green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus lunatus*), melons(*Citrullus lanatus*), peppers (*Capsicum annuum*) and many other fruits and vegetables that rapidly spoil at low, rather than high, temperature (Paull, 1990). Storage, handling and distribution of these crops are, therefore, restricted where low temperatures have deleterious effects when used to preserve quality.

Raison and Lyons (1986) refer to ‘chilling’ as the decrease in temperature treatment itself. They describe chilling as having two components: temperature and duration. Deleterious physiological changes that are inflicted by exposure to chilling temperatures are termed ‘chilling injury’. Physiological changes that are a result of chilling are further broken down into primary changes, where initial injuries are reversible upon incubation at higher temperatures, and secondary changes, where initial injuries sustained are irreversible.

‘Chilling-sensitive’ plants are those that suffer primary chilling injuries at temperatures below threshold, whereas ‘chilling-insensitive’ plants are those that do not demonstrate chilling injuries at any temperature above 0°C. These terms are distinguished from ‘chilling-tolerant’ plants, which are those that experience primary chilling injuries, yet avoid succumbing to characteristic secondary chilling injuries. Thus, if a plant suffers primary injuries upon chilling and the injuries are reversible, the plant is considered ‘chilling tolerant’, but if injuries persist, the plant is considered ‘chilling sensitive’, and if the plant suffers no injuries it is considered ‘chilling insensitive’.

2.2.1 Symptoms of chilling injury

It is not possible to immediately and unequivocally diagnose a plant as suffering chilling injury as there is no unique or characteristic change in a plant. Depending on exposure duration, temperature, time of day, nutrient availability, and other environmental conditions, visual symptoms of chilling injury vary (Saltveit and Morris, 1990). Brief chilling events often will not produce obvious symptoms while the plant is

at low temperatures, as most symptoms of chilling injury require time to appear. Clear symptoms of chilling injury are often seen when the plant is returned to optimal growth temperatures. Additionally, longer durations of low temperatures cause the symptoms of chilling injury to appear more quickly in a time-dose dependant manner. This observation is not uncommon, and implies a role of metabolism in the development of injury symptoms.

In the absence of other visual symptoms of chilling injury, chilling sensitive plants often exhibit reduced growth. These chilled plants may or may not recover normal growth rates when returned to optimal growing temperatures. For example, maize (*Zea mays*) seedlings that were chilled to 0.3 ºC for 24 h required four days before leaf expansion rates recovered to normal levels (Creencia and Bramlage, 1971). It is often the case that plant development is slowed after chilling, and plants that experience an episodic chilling event may remain visually stunted compared to non-chilled controls. In other cases, chilling sensitive plants appear not to be affected by chilling during exposure low temperatures, but symptoms of chilling injury are observed once plants are returned to warmer conditions. For example, in chilled bell pepper (*Capsicum annuum* L.) fruit, there was a much more pronounced loss in membrane glycerolipids after a 'rewarming' period following low temperature exposure than occurred during the low temperature exposure (Whitaker, 1995).

Severe chilling stress leads to cellular autolysis and senescence (Saltveit and Morris, 1990). When chilling injury is sustained in the light, it is common that leaves lose chlorophyll and appear to yellow (Matile et al., 1999). This loss of chlorophyll and yellowing is a consequence of photo-oxidation, and this phenomenon occurs less

rapidly in darkness. Chilled leaves lose cellular integrity, as tissues appear water-soaked resulting from a loss of membrane integrity that allows the leakage of cellular fluids into the apoplastic spaces (Wolfe, 1991). Plasmolysis and turgor loss under chilling conditions also imply changes to plant cellular membranes that ultimately result in failure to maintain cellular compartmentalization, resulting in solute leakage (Kratsch and Wise, 2000).

Some plants accelerate ethylene production during low temperature exposure (Corbineau et al., 1990; Field, 1984) including *Phaseolus* (Guye et al., 1987). This is not always the case, however, as cucumbers (*Cucumis sativus* L.; Wang and Adams, 1982) and pepper (*Capsicum* spp.; Leshem and Kadish, 1992) do not increase ethylene production until they are transferred to warmer temperatures. Understanding the precise role of ethylene is confounded by studies using ethylene synthesis inhibitors, which indicate that the hormone is neither a causal agent nor essential for the development of symptoms of chilling injury.

2.2.2 Effect of low temperatures on plant development

Sensitivity to chilling is dependent on the stage of the development in chilling susceptible plants. Seedlings are generally more sensitive to chilling than more mature plants. Roots are generally more sensitive to chilling than vegetative shoots, but root tissues often do not experience the same temperatures as shoot due to the insulating properties of the soil. The most sensitive organs to low temperatures are reproductive organs. An example of this is seen in soybean (*Glycine max* Merr.), as ranges of relative chilling sensitivity are seen during different stages of plant development

(Holmberg 1973). Chilling detrimentally affects flower induction, pollen production, and male fertility, especially in chilling sensitive species. An example of this particularly extreme demonstration of chilling sensitivity was observed in soybean, where the lowest temperature for fertilization and pod formation ranged from 9 ºC to 18 ºC among cultivars (Hume and Jackson, 1981), a difference which was attributed to abnormalities of the pollen that occurred at low temperatures (Lawn and Hume, 1985). This study also showed even one night of 8 ºC resulted in inhibition of pod formation in the field. Soybeans grew slowly when grown in growth cabinets set to 9 ºC day / 15 ºC night, yet most plants flowered (Musser et al., 1986). However, none of the soybeans formed seeded pods during chilling, but flowered and grew pods (albeit, smaller than normal) when transferred to greenhouse temperatures.

Beside temperature, several components of the plant's environment also influence the amount of chilling injury sustained. These include water availability, humidity, light intensity, and time of day. At low humidity, the effects of chilling are compounded by its effects on plant water relations because chilling reduces the ability of the roots to supply water (McWilliam et al., 1982) and blocks stomatal function (Guye and Wilson, 1987). High light intensities during the day provide excess excitation energy to the photosystems promoting photoinhibition and photo-oxidation (Hällgren and Öquist, 1990). Diurnal changes in chilling tolerance are well documented. For example, tomato (*Lycopersicum esculentum*) seedlings are more sensitive at the beginning of the light period, than they are at the beginning of the dark period (King et al., 1982). Fluctuations in metabolite pools, such as sucrose and glutathione (Wise and Naylor, 1987), activation of enzymes by thioredoxin (Broin et al., 2002), or gene

transcription and translation (Guy, 1999), may also play critical roles in altering the sensitivity of a cell to low temperatures.

2.2.3 Low temperatures and photosynthesis

One of the earliest responses to low temperature in plants is the inhibition of photosynthesis. In crop species, this inhibition of photosynthesis can persist for days after a brief chilling episode. For example, peanut plants required four days to return to normal chlorophyll fluorescence levels (an indication of photosynthetic efficiency) after one cool night in the field (Bell, 1993). Greater damage to photosynthesis occurs if the plant is treated to chilling under moderate to high light intensities. While low temperatures slow nearly all metabolic reactions, there are two photosynthetic reactions that are principally effected by cold: reactions involved in carbon metabolism (Huner et al., 1998), and reactions involved in stomatal closure (Mustardy et al., 1982). Accordingly, two immediate consequences of low temperature exposure are that the demand for chemical energy is reduced, and regulation of stomatal-gated water loss and gas exchange is compromised.

Reduction of stomatal apertures occurs at low temperatures, as stomatal response becomes sluggish (Guye et al, 1987). Another reason for stomatal closure is the decrease in water conductivity through root membranes (McWilliam et al., 1982). If a leaf is chilled when the stomata are open, they have a propensity to stay open, and close more slowly to curb water loss. Conversely, stomata will show a propensity to stay closed if the chilling event occurs when the stomata are already closed.

Although chilling injury in the dark is not as severe as in the light, it is significant. Most chilling events occur at night, and are a constraint in planting tropical legume crops because photosynthesis can be severely affected for several days following a single cool night (Bell, 1993). Night chilling disrupts whole chain electron transport, as thylakoid electron transport system has an increased requirement for reducing agents (Hällgren and Öquist, 1990). Damage may also occur to alternate metabolic processes, including processes involved in carbon fixation (Sassenrath et al., 1990), sugar translocations from leaves to plant resulting in feedback inhibition of photosynthesis (Bagnall et al., 1988), and shifts in water flow as a result of impaired stomatal function and reduced root conductivity (McWilliam et al., 1982).

When plants experience low temperatures, electron transport through photosystem II is inhibited, as the photosynthetic apparatus absorbs light energy exceeding amounts required for chemical energy production. Photosystem II is compromised in two ways: the water splitting mechanism is down regulated resulting from excessive trans-thylakoid proton gradient, and the D1 reaction protein is degraded (Somersalo and Krause, 1990). Photoinhibition is the term used to describe these collective compromises to photosystem II. Photoinhibition occurs within the first few hours of incubating a plant at chilling temperatures with an exposure to moderate light. Chilling tolerant plants rapidly reverse the effects of photoinhibition when transferred to optimal temperatures (Somersalo and Krause, 1989). Chilling tolerant plants in chilling temperatures also dissipate excess light energy as heat, which provides protection against high light irradiance (Barth et al., 2001).

2.2.4 Chilling injuries

Oxidative stress must be considered as a secondary response to a primary lesion resulting from chilling injury (Raison and Lyons, 1986). Superoxide species generated as a result of chilling, in part, cause secondary injuries to photosystems and membranes. A primary source of free radicals is the activation of oxygen in leaf photosystems when excess light energy is not being sequestered. The mitochondria and the plasmalemma, which are electron transport systems, may also be sources of secondary chilling injury, particularly in non-photosynthetic tissues (Purvis et al., 1995).

Symptoms of chilling injury also coincide with damage to fatty acids as a result of peroxidation (Parkin et al., 1989). Lipid peroxidation inhibits the function of membrane-bound proteins, thereby contributing to some of the visual symptoms of injury (Shewfelt and Erickson, 1991). This theory assumes that these peroxidation reactions occur conspicuously at chilling temperatures, thus the visual evidence of damage is not observed until re-warming.

Walker and McKersie (1993) performed a study comparing a chilling tolerant species of tomato (*L. hirsutum* L.) with a cultivated tomato (*L. esculentum* L., cv H722) in which they noted a reduction of antioxidants in the cultivated tomato following a 72 hour chill at 2 ^{o}C (250 μmol m^{-2} s^{-1}). The authors noted that the levels of three antioxidants, namely tocopherol, ascorbate, and total glutathione, were more reduced in *Lycopersican esculentum* than in *Linum hirsutum*. Total levels of carotenoids, ascorbate and glutathione increased in *L. hirsutum* after chilling, which is characteristic of an acclimation response. However, differences between these two species did not appear to be related to the absolute levels of antioxidants prior to chilling, as chilling appeared

to promote oxygen radical production that, in time, overwhelms the free radical scavenging mechanisms. Differences between tolerant and susceptible species, in at least one study in tomato, were not established as a result of their aptitude for scavenging free radicals, but rather their tendency to form free radicals under chilling conditions (Walker et al., 1991). In this particular study, isolated thylakoids of *L. hirsutum* were compared to those of *L. esculentum* under non-chilled and chilled conditions. Results showed that *L. esculentum* photosystems showed greater whole chain electron transport in chilled than in non-chilled controls. As seen in thylakoids isolated from *L. hirsutum*, however, chilled plants down-regulated their electron transport (Walker et al., 1991), which indicates that the plant has greater control over its photosynthetic electron transport system.

2.2.5 The freezing process

When atmospheric temperatures fall below 0^{o}C, plant tissues display an increased potential to form ice. However the point of actual ice formation within plant tissues may be several degrees lower than 0^{o}C. The reason for this is that ice requires a nucleation site for assembly of water molecules into crystalline structures (Burke and Lindow, 1990). Ice nucleation sites are typically structures that most resemble ice itself, but as temperatures fall, certain bacteria, including *Pseudomonas syringae* and *Erwinia herbicola*, have an increased likelihood that they will serve as an ice nucleator (Lindow et al., 1993). Once initial ice formation occurs, ice propagates from the initial nucleation source along newly formed ice. One strategy plants employ to survive

subzero temperatures is to tolerate ice formation on plant tissues, by controlling where ice is formed on their tissues.

Intracellular ice formation causes disruption of critical cellular processes and is generally considered lethal. Extracellular ice formation causes an increase in solute concentration and subsequently an increase in water potential. This increase in water potential causes intracellular water to flow osmotically out of plant tissues. When ice forms in the extracellular space of plant tissues, a number of physiological consequences occur, including cytoplasmic desiccation which results in an increased potential for cellular collapse and an increased potential for disruption of intercellular connections. The integrity of the plasma membrane is essential to cellular survival under these conditions, not only as a barrier between extracellular ice formation and the intracellular space, but also to keep cellular contents within the cell during ice-induced desiccation.

2.2.6 Supercooling

Most plants do not freeze at the freezing point of water, and are capable lowering the freezing point of tissue water by removing sites of ice nucleation. Lowering the freezing point of water under these conditions is known as supercooling. Supercooling is one of the main strategies employed by plants to avoid freezing so as not to endure cellular desiccation. The geographical limits of species distribution are frequently dependant upon the ability of a particular species to avoid freezing by supercooling (Burke et al., 1976).

In many herbaceous field crops, strategies aimed at avoidance of ice nucleation are ineffectual because the freezing point of soil, a potent ice nucleator, is close to 0^{o}C. To cope with extracellular freezing, these plant species control where ice-induced desiccation occurs and influence ice propagation by modifying their cell walls and producing antifreeze proteins (AFPs). AFPs are short, hydrophilic, alpha-helical proteins that infiltrate and modify the growth of ice crystals (Hon et al., 1995).

In winter rye (*Secale cereale*), plants produce both ice nucleators and AFPs aimed at directing ice propagation away from the plasmodesmata (Griffith et al., 1992). Brush et al. (1994) have suggested that the increased synthesis of ice nucleators in rye may play a role in freezing tolerance. Antibodies targeting AFPs of cold acclimated rye show that they accumulate adjacent to intercellular spaces, the epidermis (Antikainen et al., 1996), mesophyll cell walls, and xylem vessels (Pihakaskimaunsbach et al., 1996). It is thought that the localization of these AFPs in the xylem helps prevent secondary ice nucleation and propagation.

Researchers have attempted to genetically engineer freezing tolerance using an AFP isolated from flounder (*Pleuronectes americanus* Walbaum). Flounder AFP is alpha-helical in structure and is produced to facilitate fish vitality in subzero temperatures that occur in the salt water of oceans. Hightower et al. (1991) transformed the gene coding for this AFP protein into tomato and tobacco (*Nicotiana tabacum* L.) and reported that its gene product influenced *in vitro* ice formation. No reports to date have focused on the effects of transgenic AFPs *in vivo*.

2.2.7 Cold acclimation

Freezing tolerance is induced in perennial and winter annual plants by environmental signals including low temperature and/or short photoperiod which are characteristic of autumn. Plants growing during the summer are sensitive to sub-zero temperatures, but the same plant in late autumn is tolerant of prolonged exposure to freezing temperatures (Wu et al., 1999). In natural environments, plants begin to experience low but non-freezing temperatures in autumn, as temperatures tend to fall and photoperiods shorten. While photoperiod duration and temperatures required to induce an acclimation response varies from species to species, 12 hour photoperiods and 2-5^{o}C temperatures are commonly required to signal increases in freezing tolerance (Castonguay et al., 1994). In winter wheat cultivars, field trials indicate that maximal freezing tolerance is achieved in December when plants of this crop survive to -22^{o}C (Fowler et al., 1983). However, considering the natural variation of field temperatures necessary to induce maximal acclimation responses, freezing tolerance can range considerably from year to year, often by as much as 8^{o}C (Fowler et al., 1983).

Acclimation potential ranges from species to species. Winter cereals can often obtain freezing tolerance levels below -20^{o}C (Fowler et al., 1983), whereas potatoes only achieve freezing tolerance levels of around -4.8^{o}C (Seppanen et al., 1998). Tomato (*L. esculentum*) is not considered to be a freezing tolerant crop, and even cold acclimation temperature treated members of this species display lethality at -2.0^{o}C (Zhang et al., 2004).

The expression of freezing tolerance is often heterogeneous across plant tissues and organs. Potato plants (*Solanum tuberosum*) can survive a freezing event and

regrow leaves and roots so long as meristematic crowns are properly functioning and are not injured (Chen et al., 1983). Proper functioning of winter cereal crown basal meristems is critical to plant survival post-freezing, as these tissues are responsible for new root production, as plants that were frozen at -8^{o}C regrew (Tanino and McKersie, 1984). Additionally, the winter wheat crowns in these experiments responded positively, except for cells in the vascular transition zone, after being treated with the viability stain tetrazolium (Tanino and McKersie, 1984). This experiment also served as a demonstration that freezing portions of a plant can result in killing of the entire plant

2.2.8 Role of carbohydrates

The accumulation of soluble sugars is one of the earliest responses of plants when exposed to cold temperatures. Sugar accumulation in *Arabidopsis* is seen after only two hours of cold temperature incubation (Wanner and Juntilla, 1999). Soluble carbohydrates may not only function to drop the freezing point of tissue water or act as an agent to maintain osmotic balance (Levitt, 1980; Strauss and Hauser, 1986; Crowe et al., 1992; Travert et al., 1997), but these sugars may also play role in tissue nutrition during cold acclimation (Trunova, 1982) and recovery from low temperature stress (Eagles et al., 1993).

In one example, the sugar content of cabbage (*Brassica oleracea*) leaves has been positively correlated with freezing tolerance (Saskaki et al., 1996) as sucrose, glucose, and fructose gradually increased during incubation at cold acclimation temperatures (5^{o}C). Differences in freezing tolerance among cold- tolerant and cold-susceptible cultivars of alfalfa (*M. sativa*) were closely related to raffinose and

stachyose accumulation, but were not closely associated with sucrose levels (Castonguay et al., 1994).

2.2.9 Role of lipids

Plasma membrane composition is important in low temperature tolerance because it is made up of lipids that range in melting points. To explain some of the impacts of chilling injury on the plasma membrane, an elegant model was proposed by Lyons (1973). In this model, low temperature is sensed by the fatty acid tails of cell membrane phospholipid molecules such that it results in a lateral phase separation forming gel and liquid-crystalline phase domains. Gel phase domain lipids have rigidly packed acyl chains and result in membrane lipids with little kinetic motion, and thus result in improper functioning of integral membrane proteins in low temperatures. When integral membrane proteins function improperly, they fail to maintain an effective permeability barrier for cells. It is this kind of membrane breakdown that represents some of the primary effects of cellular damage due to chilling stress. Consequently, secondary effects of chilling stress follow from membrane phase breakdown, including cytosol leakage, cellular metabolic perturbation, photosynthetic disruption, and death of the cell. Raison and Lyons (1977) hypothesized that should the inner membranes of the mitochondria (cristae) suffer a lateral phase separation as a result of chilling stress, adenosine triphosphate (ATP) production would be severely compromised.

As temperatures fall, so do rates of chemical reactions and cellular respiration is not an exception. If the rate of chemical reaction decrease is exclusively the result of a

decrease in temperature, reaction rates would display a characteristic decline. However, if the chemical reaction is governed by a membrane bound protein, a characteristic slope change may be seen in the line of the Arrhenius activation energy plot, such as in hog renal brush border membrane vesicles (De and Kinne, 1981). Such changes in activation energy are linked to changes in membrane fluidity, and it is commonly believed that membrane bound proteins are affected by changes in the cell membrane lipid bilayer. For example, maize (*Zea mays* L.) root mitochondria membranes experience phase transitions at 12 and 27^{o}C and succinate oxidase, a membrane bound enzyme, experiences alterations in activation energy at the same two temperatures (Raison et al., 1977). This is one of many experiments that link temperature, membrane fluidity, and enzymatic reactivity.

A hypothesis that can be drawn from the Lyons and Raison (1970) model is that alterations in lipid phase transition would have a subsequent affect on plant chilling sensitivity. Since membrane fluidity is linked to saturation of non-polar fatty acid groups, plant biologists studying chilling sensitivity focused many of their efforts to establish a parallel between membrane fatty acid saturation and sensitivity to chilling (Kendrick and Bishop, 1986). This hypothesis was supported by evidence that plants that originate from tropical and subtropical regions had a high propensity for higher amounts of saturated fatty acids in their membrane phospholipids than membranes of plants that originate in temperate regions (Limin and Fowler, 2000). Plant species that respond to cold acclimation treatments do so by increasing the quantity of unsaturated fatty acids in their membranes (Browse and Somerville, 1991). Despite these

observations, building strong correlations using bulk lipid samples have not been established (McKersie, 1996).

Phosphatidylglycerol, a common membrane lipid, containing 16:0, 18:0, and 16:1-trans fatty acids undergo phase transition from liquid crystalline to gel phase in temperatures greater than 20^{o}C (Wu and Browse, 1995). A relationship has been seen between proportion of acyl lipid 16:1 and chilling stress upon examination of phospholipid classes of thylakoid membranes (Murata et al., 1992). Even though the relationship between chilling stress and natural acyl lipid 16:1 levels has not held across all plant species (Bishop, 1986), transgenic experiments directed towards elevating the proportions of high melting point phospholipids have established membrane fatty acid saturation levels as having a major role in chilling stress (Hamada et al., 1998). Ratios of unsaturated fatty levels have been altered by manipulating the action of acyl-lipid desaturases and glycerol-3-phosphate acyltransferase, and these alterations had corresponded changes in chilling sensitivity (Nishida and Murata, 1996). Genetic manipulation of acyl-lipid desaturases and glycerol-3-phosphate acyltransferase in tobacco and cyanobacteria has demonstrated the critical role of membrane fatty acids in protecting photosynthetic apparati from photoinhibitory effects endured in low temperature conditions (Kodama et al., 1994; 1995; Nishida and Murata, 1996). In one of the first published examples using transgenics to study the effects of membrane fatty acid saturation, Murata et al. (1992) expressed a cDNA coding for glycerol-3-phosphate acyltransferase derived from a low temperature sensitive squash (*Cucurbita moschata*) in tobacco, a species known for lacking chilling and freezing tolerance, and found that these transgenic tobacco plants were more susceptible to chilling injury than untreated

tobacco. However, when a cDNA derived from *Arabidopsis* was used to transform tobacco, the results showed that the transgenic plant increased their chilling tolerance levels. The success of these experiments in imparting chilling tolerance through the use of transgenics suggest that progress can be made using genetic engineering techniques directed towards manipulating membrane fatty acid saturation levels. While these experimental data firmly establish levels of lipid unsaturation as a major factor in the ability of plants to sense low temperatures, lipid saturation levels are not the only factor to consider in studying chilling stress.

2.2.10 Abscisic acid and low temperatures

Applying abscisic acid (ABA) to plants can induce increased freezing tolerance (Chen and Gusta, 1983). In their experiments, treating callus cultures of birdsfoot trefoil (*Lotus corniculatus* L.) with ABA for two weeks at warm temperatures resulted in increased vigor at subzero temperatures compared to non-ABA-treated controls. In these ABA-treated cultures, calli did not show signs of freezing injury until -30^{o}C while untreated calli froze at -8^{o}C. Dallaire et al. (1994) applied ABA to wheat calli, finding that these calli had freezing tolerance levels that resembled low temperature treated plants. While exogenous application of ABA resulted in increased freezing tolerance in intact wheat, intact ABA treated plants exposed to a brief period of low, but non-freezing temperatures showed the greatest increase in freezing tolerance (Dallaire et al., 1994).

One way to specifically study the role of ABA is to work with *aba1* mutants of *Arabidopsis*. Heino et al. (1990) found that *Arabidopsis* mutants lacking the ability to

synthesize ABA were unable to acclimate at low temperatures. Treating these mutants with exogenous ABA enabled these plants to recover the ability to acclimate. However, as Gilmour and Thomashow (1991) suggest, caution must be taken with interpreting results of experiments using ABA knockouts because of the pleiotropic effects of ABA in normal plant growth, noting that these mutants display severely reduced vigor and appear generally unhealthy even before cold treatments.

2.2.11 Other hormones and low temperatures

While much research has explored the role of ABA in low temperature responses in plants, other plant hormones have been shown to play a role in plant low temperature responses. For instance, Yu et al. (2001) showed that endogenous ethylene production increased significantly over the first 24 hours of low temperature exposure in winter rye. Furthermore, exogenous applications of ethylene resulted in the accumulation of apoplastic AFPs in winter rye (Yu et al., 2001). A global assay of genes up-regulated in low temperature treated *Arabidopsis* showed that their may be cross-talk between ABA and ethylene responses (Hannah et al., 2006).

The Aux/IAA family of transcription factors is up-regulated by auxin, but repress the activity of Auxin Responsive Factor (ARF) family proteins towards their target genes, which results in either activation or repression of gene transcription (Dharmasiri and Estelle, 2004). Yu et al. (2001) found that members of the Aux/IAA family were down-regulated and members of the ARF family is up-regulated in a similar manner reported in experiments using brassinosteroid treatment (Nemhauser et al., 2004). This finding suggests that Aux/IAA family of transcription factors is an

important point of cross-talk between auxin, brassinosteroids, and other signaling pathways involve in low temperature response.

2.2.12 Signaling events during low temperature exposure

Weiser (1970) speculated that the ability of plants to tolerate low temperatures required shifts in gene regulation and transcriptional modulation. Guy et al. (1985) concluded that cold acclimation corresponded with alterations in gene expression in spinach. Since then, identification and characterization of these cold responsive genes has been a major focus of many researchers. Among the key genes identified to date is fatty acid desaturase of *Arabidopsis* (*FAD8*) which has been shown to impart increased low temperature tolerance by altering the lipid profiles such that cellular membranes maintain their fluidity in low temperature conditions (Gibson et al., 1994). Genes whose products are protein chaperones, such as hsp70 gene in spinach (*Spinacia oleracea* L.) (Anderson et al., 1994) and *hsp90* in *Brassica napus* (Krishan et al., 1995), have also been found to be cold-responsive and are thought to protect proteins from denaturation at low temperatures.

Another group of genes that are expressed at low temperatures produce cold acclimation proteins (CAP) and were originally identified in spinach (Guy and Haskell, 1987). The induction of CAP genes further underscores the amount of crosstalk between different abiotic stresses and hormone treatments, as they have also been found to be up-regulated by dehydration and after application of ABA. Two CAP proteins, CAP85 and CAP160 appear to be localized in the cytoplasm, although parts of CAP160 appear to co-localize with mitochondrial fractions (Kaye et al., 1998). Kaye et al.

(1998) transformed tobacco plants with constitutively expressed spinach CAP85 and CAP160 to study their roles in freezing tolerance. Results showed that while there was little change in the temperature at which 50% electrolyte leakage occurred (EL50) compared to controls when one or both of these CAP proteins were over expressed, the rate of freezing induced membrane damage was reduced in the transgenics. Once again, the stability of cellular membranes appears to be one of the possible functions of these proteins.

Genes that are upregulated late in embryogenesis, so called Late Embryogenic Abundant (LEA) genes and their homologs, represent the largest set of cold-inducible genes. These genes are thought to protect embryos from desiccation (Ingram and Bartels, 1996). Unifying features of LEA proteins include their pronounced hydrophilicity and their repeated stretches of simple amino acid motifs that are predicted to form alpha helices.

LEA genes are quite similar to another battery of genes known to be up-regulated during low temperature incubation. *COR* (cold regulated) genes code for small and extraordinarily hydrophilic proteins (Thomashow, 1999). *COR* genes have been found to be strongly up-regulated 4-8 hours after initial chilling events (Fowler and Thomashow, 2002). Thomashow and colleagues have isolated a number of *COR* genes from *A. thaliana* (Hajela et al., 1990; Gilmour and Thomashow 1991; Gilmour et al., 1992). Northern hybridizations have indicated transcript levels of these genes increased dramatically after four hours of treatment at 5°C. These transcript levels remained high for the duration of the low temperature period, and declined after transfer to warmer temperatures (Howarth and Ougham, 1993). Many *COR* genes were also

found to be ABA-responsive as wheat plants accumulated *COR* transcripts when sprayed with ABA at room temperatures (Guo et al., 1992). Furthermore, expression of *COR* genes can be brought about by drought stress but not by heat shock (Hajela et al., 1990). The peptides from these *COR* genes are stable and remain stable after boiling (Thomashow, 1999).

Despite the knowledge of many of the unique properties of *COR* genes, their function with regard to freezing tolerance is, to date, unknown. One hypothesis is that COR proteins serve a cryoprotective function. In testing this hypothesis, Lin and Thomashow (1992) found that *Arabidopsis* COR15 protein was about 10^6 times more effective than sucrose in protecting the cold-labile enzyme lactate dehydrogenase from inactivation in freezing temperatures (Lin and Thomashow, 1992).

Two COR peptides of note have been studied in greater detail in *Arabidopsis*. The COR15a polypeptide is targeted to chloroplasts and, during import, is processed to a 9.4-kDa polypeptide COR15am which subsequently fuses to the membrane of the chloroplast (Steponkus et al., 1998). Another COR polypeptide, COR6.6, is located in the cytosol (Gilmour et al., 1996). Neither COR6.6 nor COR15am influenced freezing-induced leakage of small unilamellar vesicles (Uemura et al., 1996), and neither COR6.6 nor COR15am influenced the temperature at which membrane proteins dipalmitoylphosphatidylcholine and dioleoylphosphatidylcholine undergo gel to liquid crystalline phase transition, which suggests that neither of these COR polypeptides participate in specific protein-phospholipid interactions (Webb et al., 1996).

A cold-inducible promoter has been found for *Arabidopsis COR15a*. The 5' region of *COR15a* at the relative transcriptional start site contains a cis-acting

element(s) that can impart cold-regulated gene expression (Baker et al., 1994). This promoter is non- or very weakly active in most of the tissues and organs of plants grown at normal temperature (Baker et al., 1994). However, this promoter region becomes activated throughout most of the plant in response to low temperature (Baker et al., 1994). Additionally, this promoter responds to abscisic acid (Hoth et al., 2002) and drought (Sakuma et al., 2006). Low temperature regulation of COR15a does not involve the synthesis of a regulatory molecule that can spread throughout the plant and induce *COR* gene expression at normal growth temperature (Steponkus et al., 1998).

2.2.13 Low temperature responsive transcriptional regulators

In order for plants to survive low temperature stress, they require alterations in gene regulation to properly produce proteins and metabolites necessary to mitigate the damaging effects of low temperature exposure. Additionally, plants often modulate genes that function in cell signal transduction in response to low temperatures in order to facilitate appropriate low temperature responses. Proteins commonly referred to as transcription factors function as master switches to batteries of downstream genes whose functions may vary but whose purpose ultimately impact appropriate responses to stress. Transcription factors bind to promoter sequences upstream of genes, and this DNA binding action facilitates the expression of these genes. In recent years, researchers have targeted much of their efforts towards understanding the regulation and function of these master switches when trying to ameliorate the effects of stress.

Transcription factors that have been shown to be responsive to low temperatures are members of the group APETALA2 (AP2) (for review, see Nakashima and

Yamaguchi-Shinozaki, 2006). AP2 transcription factors were initially characterized for their role in the promotion of the establishment of the floral meristem and regulation of floral organ development in *Arabidopsis* (Jofuku et al., 1994). Cheong et al. (2002) found that members of the AP2-type transcription factor family, including CBF (C-repeat binding factor) and RAV (Related to ABI3/VP1) were induced upon wounding in *Arabidopsis*. CBF-type transcription factors have been widely studied for their role in low temperature response as well as drought response (for review see Shinozaki and Yamaguchi-Shinozaki, 2000).

Stockinger et al. (1997) characterized the AP2-type transcription factor CBF1 as weighing 24 kDa and containing an AP2 domain flanked by signature amino acid sequences PKK/RPAGRxKFxETRHP and DSAWR. The AP2 domain has been characterized in *N. tabacum* by Ohme-Takagi and Shinshi (1995) as having DNA binding properties, and AP2-type transcription factors have been found in many plant species. Furthermore, tobacco EREBP (Ethylene Response Element Binding Protein) binds to the promoter element AGCCGCC, which strongly resembles the dehydration responsive element (DRE). DRE contains the TACCGACAT core sequence shown to be linked to dehydration, low temperature, and high salt responses, as well as abscisic acid treatment (Yamaguchi-Shinozaki and Shinozaki, 1994). The close resemblance of the EREBP and the DRE led Stockinger (1997) to suggest that these EREBPs along with CBF, and likely other AP2 proteins, belong to a 'superfamily' of similar proteins that bind *cis*-acting elements that contain a common CCG core sequence. Since the initial cloning and characterization of CBF1, further investigations have lead to the discovery of more CBF genes in *Arabidopsis*, namely CBF2 (Gilmour et al., 1998),

CBF3 (Liu et al., 1998), and CBF4 (Haake et al., 2002). This group represents a CBF gene family. *CBF*-like genes have also been identified in alfalfa (*M. sativa*; Zhang et al., 2004), *B. napus* (Jaglo et al., 2001); rye (*Secale cereale*; Jaglo et al., 2001), wheat (*Triticum* spp.; Jaglo et al., 2001), tomato (*L. esculentum*); (Jaglo et al., 2001), rice (*Oryza sativa*; Dubouzet et al., 2003), and *N. tabacum*; (Zhang et al., 2004), among other species.

Insights into the up-regulation of *CBF* genes are emerging. Shinwari et al. (1998) established the presence of low temperature responsive *CBF* promoters, and Chinnusamy et al. (2003) identified a low temperature activated MYC-like basic helix-loop-helix transcription factor, termed ICE (Inducer of CBF Expression), which enhances low temperature expression of CBF in *Arabidopsis*. ICE and low temperature mediated CBF expression up-regulates *LEA*/*COR* genes as described above, resulting in cold acclimation (Figure 2.1)

Strong up-regulation of *Arabidopsis CBF* genes occurs within the first hour after transferring plants to low temperature. This up-regulation is followed by increased transcript levels of *COR* genes after around two hours of sustained CBF expression (Gilmour et al., 1998; Liu et al., 1998). Jaglo et al., (2001) assayed a variety of species with a range of freezing tolerance levels and found that species with the greatest ability to cold acclimate (and thus greatest freezing tolerance) tended to have more rapid *CBF* transcript accumulation as well as greater overall CBF expression. Using a transgenic approach, Jaglo-Ottoson et al. (1998) found that over expression of CBF1 resulted not only in increased freezing tolerance in *Arabidopsis*, but in addition, in an increased ability to withstand drought stress. Shinozaki and Yamaguchi-Shinozaki (2000) found

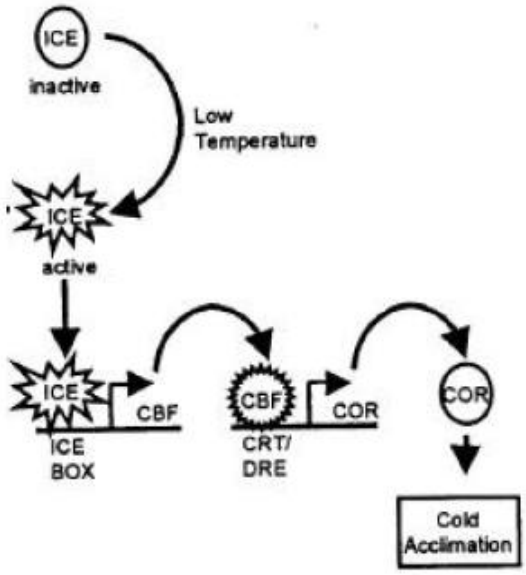

Figure 2.1. Schematic illustration of cold regulated gene expression in plant tissues.

ICE (inducer of CBF expression) is activated by cold into its active form and subsequently interacts with an ICE-box promoter region of *CBF* (C-repeat binding factor). CBF proteins, in turn, bind to the CRT (C-repeat) promoter region of *COR* (cold regulated) and other low temperature responsive genes, resulting in cold acclimation. Adapted from Thomashow (1999).

that the promoter region of *COR78* contained the CCGAC core sequence of the 9-bp *DRE* (dehydration responsive element), TACCGACAT, and was responsive to increased amounts of salinity in addition to cold and dehydration. Taken together, these results not only underscore the link between abiotic stresses of cold, drought, and high salinity, but also link CBF regulation to the amelioration of these stresses.

Since there is considerable cross-talk between ABA-dependant and independent signaling networks in response to low temperatures (see Shinozaki and Yamaguchi-Shinozaki, 2000, for review), RAV-type transcription factors represent an interesting angle in studying plant stress responses. As mentioned previously, RAV transcription factors have both B3 and AP2-type DNA binding motifs. In *Brassica napus*, proteins with B3 DNA-binding domains bind to DNA in an ABA-dependant manner (Ezcurra et al., 2000), while proteins with AP2 DNA-binding domains bind to DNA in low temperature conditions in an ABA-independent manner in *Arabidopsis* (Thomashow, 1999). Thus, RAV transcription factors have the potential to govern gene signaling responses under a variety of stresses.

2.3 Using array technology to study effects of low temperatures

Array technology is widely recognized for its ability to potentially provide genome-wide information regarding gene expression patterns. Use of array technology is appealing to many researchers because it enables the profiling of large numbers of genes in a single experiment. Array technology has been used to study plant responses to a variety of treatment conditions, including ABA treatment (Seki et al., 2002), rehydration (Oono et al., 2006), reactive oxygen species treatment (Narasuka et al.,

2004), jasmonic acid application (Narasuka et al., 2004), ethylene application (Narasuka et al., 2004), and salicylic acid application (Narasuka et al., 2004). Additionally, cold, drought, and high-salinity stresses have been extensively studied using microarray technology (Shinozaki and Yamaguchi-Shinozaki, 2000; Seki et al., 2002; Xiong et al., 2002; Zhu, 2002; Sharma et al., 2007).

Although many genes have been found to be transcriptionally responsive to abiotic stress, a great many of these genes have yet to be identified. In an *Arabidopsis* microarray study featuring 1300 full-length cDNAs, Seki et al. (2001) identified 44 drought-inducible and 19 cold-inducible genes. Of the 44 drought-inducible genes, 30 had not yet been previously identified as drought-inducible, and 10 of the 19 cold-inducible genes had not yet been previously identified as cold-inducible. Using cDNAs derived from *Arabidopsis* over-expressing *CBF3* and an *Arabidopsis* full-length cDNA microarray containing 1300 cDNAs, Seki et al. (2001) identified six novel *CBF3* induced genes. All of these *CBF3* target genes had either the *DRE* (Yamaguchi-Shinozaki and Shinozaki, 1994) or some variation of the CCGAC core motif in their promoter regions (Seki et al., 2001). In a more recent experiment, cDNAs were derived from drought, cold, high-salinity, and ABA treated *Arabidopsis* and hybridized to a microarray featuring 7000 full length *Arabidopsis* cDNAs to identify stress-specific inducible genes (Seki et al., 2002). Using this approach, 299 drought-inducible genes, 54 cold-inducible genes, 213 high-salinity-stress inducible genes, and 245 ABA-inducible genes were identified. Furthermore, Venn diagram analysis indicated significant cross-talk between these stresses (Seki et al., 2002). Overall, these findings show the potential that array technology demonstrates with regard to understanding

plant responses to stress, as indicated by the identification of the considerable overlap between these abiotic treatments and stresses.

2.3.1 Use of related species to conduct functional genomics experiments

With the advent of the genomic era, researchers for some crop species, like *Phaseolus,* are lacking resources to study responses to low temperatures. Advances in genomic comprehension are currently limited to those species that have the available genomic resources, which commonly include genome sequencing, cDNA libraries, and microchips printed with nucleic acids of known sequence. In the case of the plant kingdom, *A. thaliana* has emerged as the model plant species to study transcriptional alterations on a genomic scale. Other plant species that have well developed microarray platforms, including barley (*Hordeum vulgare*), rice (*O. sativa*), maize (*Zea mays*), tomato (*L. esculentum*), sugar cane (*Saccharum officinarum*), grape (*Vitis vinifera*) and wheat (*Triticum aestivum*) (Hammond et al., 2006). An underlying assumption when conducting such arrays is that there sufficient similarity between the nucleic acid sequences of the genomes being assayed.

In legumes, a cDNA library has been constructed from cold acclimated *M. sativa* (Castonguay et al., 1994). Since the *Phaseolus* genus currently lacks sufficient resources to conduct genome wide profiling of transcripts, cross-species hybridization using the legume *M. sativa* cDNAs provides an interesting and possible opportunity to study some degree of transcriptional alterations in common bean. Alfalfa appears to be a strong candidate for such cross-species studies with bean due to known phylogenetic relationships between *Medicago* and *Phaseolus*, as taxonomic analyses show they both

belong to the Papilionoideae subfamily of flowering plants which diverged from other Fabaceae 45-50 million years ago (Choi et al., 2004). DNA sequence alignments of *M. sativa* with *P. vulgaris* and *P. angustissimus* tend to distribute around the 86-97% sequence identity mark, and thus are presumably orthologues. In light of recent successes seen in cross species hybridization among close relatives of *Arabidopsis* using transcripts of *Arabidopsis halleri*, *Brassica napus*, *Thlaspi arvense***,** and *Thellungiella halophila*, the possibilities of using such approaches to study stress responses in species lacking genomic resources has become a possibility (Becher et al., 2004; Lee et al., 2004; Taji et al., 2004; Gong et al., 2005; Sharma et al., 2007). Thus, a near-term solution for assaying transcripts of novel species can be undertaken via cross-species arrays.

3. Physiological effects of low temperature exposure in *Phaseolus* species

3.1 Introduction

Seedlings of many tropical species cannot survive prolonged exposure to low temperatures. The threat of chilling injury restricts growth of susceptible crops to specific geographical regions that have low probabilities of experiencing damaging temperatures. Potentially damaging chilling conditions are commonly experienced by crop species on cool spring mornings in temperate zones. Nonetheless, some degree of chilling tolerance is exhibited by some chilling-sensitive species, as indicated by the results of experiments with hardy plants (Perez de Juan et al., 1997) or with different genotypes of maize (Capell and Dorffling, 1993) and rice (Lee et al., 1993) and some *Phaseolus* species (Balasubramanian et al., 2004).

The capacity of plants to demonstrate tolerance to chilling temperature varies among genotypes and species. When chilling temperatures are experienced, it is critical for plants to avoid damage to their photosynthetic apparatus. In addition, the ability to maintain a favourable water status is an important component of plant tolerance to chilling, as one of the first symptoms of chilling injury is dehydration caused by the imbalance between root water uptake and leaf transpiration (Sanders and Markhart, 2001; Vernieri et al., 2001).

The exact level of chilling injury is dependant on which species is being exposed to chilling temperatures. Visual symptoms of chilling injury vary, depending on the temperature, duration of exposure, stage of development and tissue type, the time of day and other environmental conditions such as light, wind, water and nutrients (Saltveit and Morris, 1990). Most symptoms require time to appear. Although a brief chilling event will not produce immediate obvious symptoms, they do gradually appear afterwards, especially when the plant is returned to optimal growth temperatures. Prolonged exposure to low temperatures results in visible symptoms of chilling injury.

Plants that experience chilling may exhibit a loss of vigor and reduced growth rates in the absence of other visual symptoms of injury. In addition, inhibition of photosynthesis is an early response to low temperature (Stitt and Hurry, 2002). Whereas arctic and alpine plants conduct measurable photosynthesis at temperatures near or just above 0°C, the growth of most chilling-sensitive crop plants is severely inhibited at these temperatures, and this inhibition can last for days after the chilling event.

The effects of chilling temperatures on photosynthesis are much more severe if exposure occurs coincident with moderate or high light intensities. Low temperatures cause nearly all metabolic reactions to slow. In terms of photosynthesis, there are two metabolic reactions that are particularly sensitive to low temperatures: those involved in CO_2 fixation, and those involved in regulating stomatal aperture (Farquhar and Sharkey, 1982). Consequently, two immediate consequences of exposure of leaves to low temperature are that the

demand for chemical energy is reduced, and that the ability of the stomata to regulate water loss and CO_2 exchange is altered (Farquhar and Sharkey, 1982).

Many plants acquire greatly improved freezing tolerance during exposure to low, nonfreezing temperatures in a process called cold acclimation (Levitt, 1980; Guy, 1990). In the laboratory, freezing tolerance can be induced by acclimating plants at 2 to 5^{o}C over a period of days or weeks. During cold acclimation, a variety of metabolic changes occur and specific genes are expressed (Guy, 1990). While cold-induced genes have been isolated and their expression characterized in several plant species (see Guy, 1990, and Thomashow, 2001), the mechanisms by which low-temperature signals are perceived and transduced into biochemical responses are still not known.

A previous study indicated that common bean (*Phaseolus vulgaris* L. cv. ICA Pijao) has very little freezing tolerance in both environmental and controlled conditions (Balasubramanian et al., 2004). Balasubramanian et al. (2004) also identified a wild bean species, *Phaseolus angustissimus* L., which demonstrated a greater ability to survive sub-zero temperatures. In order to compare these two species with regard to their respective responses to low temperatures, plants of each species were evaluated with regard to their response to a cold acclimation treatment as measured by survival in freezing conditions. As well, chilled plants of each species were compared to appropriate non-chilled controls over the course of five days with regard to alterations in plant height, plant weight, chlorophyll content, and chlorophyll a/b ratios. The wild species demonstrated marginally better freezing tolerance after three days of chilling

temperatures compared to the cultivated species, and less dehydration of aerial tissues over the first three days of chilling. Plants of both species demonstrated impaired growth rates with little difference in total chlorophyll quantities. Evidence is presented supporting the hypothesis that the wild species suffers less damage as a result of low temperatures, identifying it as a potential source of genetic material to incorporate chilling tolerant phenotypes in the cultivated bean species.

3.2 Materials and methods

3.2.1 Plant material and growth conditions

Phaseolus vulgaris L. cv. ICA Pijao and the wild bean, *Phaseolus angustissimus* L. PI 535272 obtained from the USDA National Plant Germplasm System, were grown until emergence in Jiffy peat pellets (Novosel Enterprises, Oberlin, Pennsylvania, USA) in a controlled environment growth cabinet with an alternating temperature regime of 23 ^{o}C day / 18 ^{o}C night with a 16 hour photoperiod and photosynthetic photon flux density (PPFD) of 250 μmol m^{-2} s^{-1}. After emergence, seedlings were transplanted to Styrofoam (The Dow Chemical Company, Midland, Michigan, USA) boxes, four per box (planting area of 22 cm long, 18 cm wide, 18 cm deep, with 6 cm of insulation) filled with soil, where they continued to grow for the remainder of the experiments. Styrofoam boxes were used to protect the roots from cold air and to attempt to prevent their premature freezing. To expose plants to low temperatures, representative plants of each species were transferred to a growth cabinet set to 7 ^{o}C day / 5 ^{o}C night, featuring

the same PPFD and duration as the warm growth cabinet, as described below. Plants were watered as needed.

3.2.2 Tolerance to sub-zero temperatures

The ability of plants to tolerate sub-zero temperatures was determined by their ability to survive decreasing temperatures after being subjected to three days of incubation in chilling conditions. Six boxes (four plants per box for a total of 24 plants) of each species were transferred to a cabinet pre-set to -1 ^{o}C at subjective nightfall, ice nucleated with a fine spray of water, and incubated for 1 hour. After this 1 hour exposure, one box (four plants) of each species was returned to the chilling cabinet. The remaining sub-zero treated plants remained in the freezing cabinet and the temperature was dropped 0.5 ^{o}C over the course of 0.5 hour. Plants were held for 1 hour at -1.5 ^{o}C after which time one box (four more plants) of each species was returned to the chilling cabinet. This procedure was repeated at each 0.5 ^{o}C interval until -3.5 ^{o}C, at which point all plants had been returned to the chilling cabinet. All plants subjected to freezing treatment were maintained at chilling temperatures for 12 hours, then transferred to 23 ^{o}C / 18 ^{o}C for 24 hours and visually assessed for their ability to survive each temperature, noting severe dehydration and drastic loss of healthy green leaves as characteristic symptoms of plant lethality. This experiment was repeated four times and the percent survival at each temperature was determined based on mortality within all 16 plants per species at each temperature. In total, 96 plants (four plants in each of six temperature intervals repeated four times) of each species were subjected to cold acclimation experiments.

3.2.3 Chilling tolerance

Response of each species to chilling temperatures was determined by comparing chilled plants to their age-matched non-chilled controls. Eight plants of each species were grown in Styrofoam boxes (planting area of 44 cm long, 36 cm wide, 25 cm deep, with 6 cm of insulation) and half the experimental population of each species was subjected to chilling temperatures as described in 3.2.2 above. The day after subsets of each species were transferred to the chilling cabinet, four plants of each species in each condition (growth and chilled) were measured for height and fresh weight. After height and weight measurements, the plants were randomly separated into two representative sub-samples; one sub-sample was used to calculate dry weight, which was measured after plants were subjected to a drying oven set to 75 ^{o}C until they were dry. A 0.25 g sample of leaf tissue from the second sub-sample was flash frozen in liquid nitrogen and stored at -80 ^{o}C for chlorophyll extraction. This method of harvesting and measurement was done for each of the subsequent four experimental days with living plants being sacrificed for measurements each day. The number of plants of each species sampled each day (4) used for these experiments was small, however, the number of plants used as a representative sample in these experiments is consistent with many previously published studies of low temperature exposure on plants using controlled environments (see Walker et al., 1991; Wu et al., 1997; Allen et al., 2000; Melkonian et al., 2004; Zhou et al., 2007).

3.2.4 Water content analysis

Sub-samples used to calculate % water content were treated as follows: samples from four plants in each species at each condition each day were weighed for fresh weight. These samples were then subjected to at least 72 hours in a drying oven set to 75 ^{o}C, after which each sample was weighed again to obtain a dry weight for each sample. From these values, percent water content values were calculated for each sample using the following formula:

((Sample fresh weight – Sample dry weight)/Sample fresh weight) x 100 (i)

3.2.5 Photosynthetic pigment analysis

Total chlorophyll was extracted from frozen 0.25 g leaf samples of four plants of each species in non-chilled and chilled conditions at each time point using 1 mL 80% acetone. Chlorophyll a and chlorophyll b contents were determined using absorbance values generated from a spectrometer (Agilent Technologies, Wilmington, Delaware, USA) and analyzed using UV ChemStation software (Agilent Technologies, Wilmington, Delaware, USA) as described in Lichtenthaler and Welburn (1983):

Chlorophyll **a** (μg/mL) = 12.21 (A_{663}) – 2.81 (A_{646}) (ii)

Chlorophyll **b** (μg/mL) = 20.13 (A_{646}) – 5.03 (A_{663}) (iii)

3.3 Results

3.3.1 Tolerance to sub-zero temperatures in *P. vulgaris* and *P. angustissimus*.

When *P. vulgaris* and *P. angustissimus* plants were grown for 21 days and subjected to 72 hours of 7 ^{o}C day / 5 ^{o}C night followed by a freezing tolerance test, there were marginal differences in survival. When *P. vulgaris* seedlings were subjected

to the freezing test, 6.3 % did not survive -1.5 ^{o}C, 37.5% did not survive -2.0 ^{o}C, and none were able to survive to -2.5 ^{o}C (Table 3.1). Comparatively, all *P. angustissimus* plants survived temperatures of -1.5 ^{o}C, 75% survived to -2.0 ^{o}C, 31.3% survived exposure to -3.0^{o}C, and some *P. angustissimus* plants managed to survive exposure to -3.5 ^{o}C (Table 3.1). The greatest contrast in tolerance was observed between the two species after decreasing temperature to -2.5 ^{o}C, where none of the *P. vulgaris* seedlings survived while 68.8% of the *P. angustissimus* seedlings continued to survive (Table 3.1, Figure 3.1).

3.3.2 Physiological responses to chilling stress

Plants of both species appeared to halt normal growth rates upon exposure to chilling temperatures (7 ^{o}C/5 ^{o}C), as neither plant species significantly increased plant height over the course of five days of chilling compared to their age-matched non-chilled controls. *Phaseolus vulgaris* plants grown in the control cabinet grew from an average height of 11.3 cm on Experiment Day 1 to an average height of 21.0 cm on Experiment Day 5, while the average height of plants transferred to a chilling cabinet remained essentially unchanged from Experiment Day 1 to Experiment Day 5 (Table 3.2). Similarly, *P. angustissimus* plants grown in the control cabinet grew from an average height of 11.5 cm on Experiment Day 1 to an average height of 17.4 cm on Experiment Day 5, while the average height of plants transferred to a chilling cabinet remained essentially unchanged from Experiment Day 1 to Experiment Day 5 (Table 3.2). By expressing each species average plant height values as ratio of age matched non-chilled control values, the two species can be directly compared. Chilled plants of

Table 3.1. Survival of *P. vulgaris* and *P. angustissimus* following exposure to sub-zero temperatures.

Temperature	*P. vulgaris*		*P. angustissimus*	
	# dead plants (/16)	%	# dead plants (/16)	%
-1.0	0	0	0	0
-1.5	1	6.3	0	0
-2.0	6	37.5	4	25
-2.5	16	100	5	31.3
-3.0	16	100	11	68.8
-3.5	16	100	14	87.5

All plants were grown for 21 days (23 ^{o}C day / 18 ^{o}C night), incubated for three days at 7 ^{o}C day / 5 ^{o}C night, and ice nucleated at -1.0 ^{o}C. The temperature was then reduced to -1.5 °C over a period of 1 hour. The temperature remained at -1.5 ^{o}C for 1 hour then was decreased by 0.5 °C and left for a further 1 hour at this new temperature. This cycle was repeated until the temperature had reached -3.5 ^{o}C. Plant samples were removed at the end of each cycle and allowed to recover for 12 hours at acclimation temperatures followed by 24 hours at growth temperatures, and observations of survival were conducted.

A

B

Figure 3.1. Photos showing response to freezing of *P. vulgaris* (left) and *P. angustissimus* (right) plants.

(*A*) Plants grown for 21 days at 23 ^{o}C day / 18 ^{o}C night, cold acclimated for three days at 7 ^{o}C day / 5 ^{o}C night, and ice nucleated at -1.0 ^{o}C. (*B*) Plants grown under same conditions following exposure to one hour at -2.5 ^{o}C.

Table 3.2. Effect of chilling in *P. vulgaris* and *P. angustissimus* on average plant height.

	P. vulgaris average height (cm+SD)			*P. angustissimus* average height (cm+SD)		
	23/18 °C	7/5 °C	7/5 °C / (23 °C) Ratio	23/18 °C	7/5 °C	7/5 °C / (23 °C) Ratio
Day 1	11.3 (3.3)	8.8 (1.0)	0.8	11.5 (1.5)	9.5 (0.7)	0.8
Day 2	12.0 (1.1)	9.5 (1.9)	0.8	12.6 (1.9)	9.8 (0.6)	0.8
Day 3	12.5 (2.0)	8.5 (1.5)	0.7	13.8 (2.4)	10.3 (2.6)	0.7
Day 4	12.3 (2.3)	8.3 (1.6)	0.7	17.9 (1.9)	9.1 (0.9)	0.5
Day 5	21.0 (9.8)	7.9 (1.4)	0.4	17.4 (3.3)	9.3 (1.4)	0.5

All plants were grown for 21 days in 23 °C day / 18 °C night temperatures. A subset of each species was transferred to a chamber set to 7 °C day / 5 °C night. Each day after transfer, average height was calculated for four warm and four chilled plants of each species. Means, standard deviations, and ratios are presented.

Table 3.3. Effect of chilling in *P. vulgaris* and *P. angustissimus* on average plant fresh weight.

	***P. vulgaris* average fresh weight (g±SD)**			***P. angustissimus* average fresh weight (g±SD)**		
	23/18 °C	**7/5 °C**	**(7/5 °C) / (23/18 °C) Ratio**	**23/18 °C**	**7/5 °C**	**(7/5 °C) / (23/18 °C) Ratio**
Day 1	3.34 (0.90)	2.98 (0.12)	0.9	0.62 (0.29)	0.42 (0.08)	0.7
Day 2	3.82 (1.16)	3.30 (1.16)	0.9	0.68 (0.16)	0.53 (0.11)	0.8
Day 3	3.10 (0.77)	2.91 (0.58)	0.9	0.70 (0.07)	0.52 (0.15)	0.7
Day 4	5.29 (1.33)	3.31 (0.98)	0.6	0.93 (0.28)	0.58 (0.19)	0.6
Day 5	7.81 (1.45)	2.85 (0.49)	0.4	0.86 (0.24)	0.52 (0.22)	0.6

All plants were grown for 21 days in 23 ^{o}C day / 18 ^{o}C night temperatures. A subset of each species was transferred to a chamber set to 7 ^{o}C day / 5 ^{o}C night. Each day after transfer, average fresh weight was calculated for four warm and four chilled plants of each species. Means, standard deviations, and ratios are presented.

Table 3.4. Effect of chilling in *P. vulgaris* and *P. angustissimus* on average plant dry weight.

	***P. vulgaris* average dry weight (g±SD)**			***P. angustissimus* average dry weight (g±SD)**		
	23/18 °C	**7/5 °C**	**(7/5 °C) / (23/18 °C) Ratio**	**23/18 °C**	**7/5 °C**	**(7/5 °C) / (23/18 °C) Ratio**
Day 1	0.68 (0.26)	1.61 (0.37)	2.4	0.09 (0.02)	0.08 (0.01)	0.9
Day 2	0.91 (0.31)	1.66 (0.39)	1.8	0.11 (0.03)	0.13 (0.01)	1.2
Day 3	0.77 (0.36)	1.23 (0.40)	1.6	0.11 (0.03)	0.11 (0.03)	1.0
Day 4	0.79 (0.24)	0.62 (0.15)	0.8	0.17 (0.04)	0.12 (0.03)	0.7
Day 5	1.19 (025)	0.56 (0.13)	0.5	0.15 (0.04)	0.14 (0.05)	0.9

All plants were grown for 21 days in 23 ^{o}C day / 18 ^{o}C night temperatures. A subset of each species was transferred to a chamber set to 7 ^{o}C day / 5 ^{o}C night. Each day after transfer, average dry weight was calculated for four warm and four chilled plants of each species. Means, standard deviations, and ratios are presented.

both species were shorter than their non-chilled age matched control in terms of plant height (Table 3.2).

There was a contrast between the two species with regard to their respective average percent water contents in the chilling cabinet, particularly from Experiment Day 1 to Experiment Day 3. *Phaseolus vulgaris*, plants grown in the control cabinet ranged from 74.8 to 85.2 average percent water content over the five Experiment Days, but their chilled counterparts had average percent water content values of 45.7, 47.5, and 58.5 on Experiment Days 1, 2, and 3, respectively (Table 3.5). On Experiment Days 4 and 5, chilled *P. vulgaris* plants had average percent water content similar to those (80.8 and 80.4, respectively) of their age matched non-chilled controls (Table 3.5). Chilled *P. angustissimus* plants did not lose water content over the five Experiment Days. Average percent water content of chilled *P. angustissimus* average plants ranged from 72.7 to 80.5 compared to their non-chilled controls which ranged from 82.0 to 84.8 percent average water content (Table 3.5). Expressing each species average plant water content as the ratio of age matched non-chilled control values, the two species can be directly compared, and this comparison shows how chilled *P. vulgaris* plants suffered a decrease in percent water content over the Experimental Days 1, 2, and 3, whereas *P. angustissimus* did not suffer a lose water content over the course of five Experiment Days (Table 3.5). The decrease in water content observed in *P. vulgaris* is reflected in the accumulation of average dry weight over the first three days of chilling (Table 3.3 and 3.4), and by Experiment Day 4, *P. vulgaris* dry weight averages were closer the average dry weights of non-chilled *P. vulgaris*.

Table 3.5. Effect of chilling in *P. vulgaris* and *P. angustissimus* on average percent plant water content.

	***P. vulgaris* average water content (%±SD)**			***P. angustissimus* average water content (%±SD)**		
	23/18 °C	**7/5 °C**	**(7/5 °C) / (23/18 °C) Ratio**	**23/18 °C**	**7/5 °C**	**(7/5 °C) / (23/18 °C) Ratio**
Day 1	80.0 (2.5)	45.7 (14.0)	0.6	82.4 (7.4)	80.5 (4.9)	1.0
Day 2	74.8 (10)	47.4 (11.3)	0.6	83.9 (0.7)	75.0 (3.7)	0.9
Day 3	75.7 (8.7)	58.5 (5.7)	0.8	84.8 (2.1)	79.4 (2.3)	0.9
Day 4	85.2 (1.6)	80.8 (3.0)	0.9	82.0 (3.1)	78.1 (2.9)	1.0
Day 5	84.8 (0.4)	80.4 (2.2)	0.9	82.9 (2.3)	72.7 (3.9)	0.9

All plants were grown for 21 days in 23 ^{o}C day / 18 ^{o}C night temperatures. A subset of each species was transferred to a chamber set to 7 ^{o}C day / 5 ^{o}C night. Each day after transfer, average water content was calculated for four warm and four chilled plants of each species. Means, standard deviations, and ratios are presented.

Table 3.6. Effect of chilling in *P. vulgaris* and *P. angustissimus* on average plant chlorophyll content.

	***P. vulgaris* average chlorophyll content (µg/g FW ± SD)**			***P. angustissimus* average chlorophyll content (µg/g FW ± SD)**		
	23/18 °C	**7/5 °C**	**(7/5 °C) / (23/18 °C) Ratio**	**23/18 °C**	**7/5 °C**	**(7/5 °C) / (23/18 °C) Ratio**
Day 1	38.7 (4.2)	48.2 (7.7)	1.2	45.1 (5.9)	53.1 (3.0)	1.2
Day 2	36.0 (7.6)	32.4 (8.5)	0.9	37.2 (4.9)	41.7 (7.5)	1.1
Day 3	34.0 (6.6)	19.7 (2.4)	0.6	43.3 (6.5)	41.8 (7.6)	1.0
Day 4	42.0 (10.0)	30.6 (5.5)	0.7	49.4 (9.0)	53.5 (4.1)	1.1
Day 5	49.6 (14.3)	46.0 (16.7)	0.9	46.4 (13.1)	49.7 (11.1)	1.1

All plants were grown for 21 days in 23 ^{o}C day / 18 ^{o}C night temperatures. A subset of each species was transferred to a chamber set to 7 ^{o}C day / 5 ^{o}C night. Each day after transfer, average chlorophyll content was calculated for four warm and four chilled plants of each species. Means, standard deviations, and ratios are presented.

There was a contrast between the two species with regard to their respective total chlorophyll content in the chilling cabinet, particularly on Experiment Day 3. The *Phaseolus vulgaris* plants grown in the control cabinet had ranges in average total chlorophyll content from 34.0 to 49.6 μg/g over the five Experiment Days, but their chilled counterparts had average total chlorophyll content of 19.7 μg/g on Experiment Day 3 (Table 3.6). The low total chlorophyll concentration on Experimental Day 3 in *P. vulgaris* was the low point in an overall trend of decrease and recovery of average total chlorophyll content over the five Experimental Days. This overall trend of decreasing average total chlorophyll content until Experiment Day 3 followed by recovery on Experiment Days 4 and 5 was also seen in *P. angustissimus*, but to a lesser extent (Table 3.6). The average total chlorophyll content of *P. angustissimus* were slightly greater than that of their age matched non-chilled controls over the five Experiment Days, with the exception of Experiment Day 3. For *P. vulgaris* average total chlorophyll content was lower than their age matched non-chilled controls on each Experiment Day except for Experiment Day 1. Expressing each species average total chlorophyll values as ratio of age matched non-chilled control values, the two species can be directly compared, and this comparison shows how chilled *P. vulgaris* plants suffered a decrease in total chlorophyll content that is most evident on Experiment Day 3, while *P. angustissimus* does not suffer a similar decrease over the course of the five Experiment Days (Table 3.6).

Chilled plants of both species closely resembled their non-chilled age match controls with regard to chlorophyll a/b ratio over the five Experiment Days. The *P. vulgaris* and *P. angustissimus* plants showed average chlorophyll a/b ratios ranging

from 0.9 to 1.7 (Table 3.7) and 0.9 to 1.6 (Table 3.7), respectively, over the five Experiment Days across non-chilled and chilled conditions. The lone exception to the pattern of chlorophyll a/b ratios was that for the chilled *P. vulgaris* on Experiment Day 3, which had an average chlorophyll a/b ratio of 3.0 (Table 3.7).

Images of these plants taken on Experiment Day 3 show the contrast between chilled plant of each species and their non-chilled controls (Figure 3.2). Chilled plants of both species appeared smaller with a lighter shade of green to their leaves compared to their respective non-chilled controls. Extra plants were left in the chilling cabinet for further observation. Two nine-day chilled plants of each species were returned to growth (23 ^{o}C day / 17 ^{o}C night) conditions and monitored, while other plants of each species remained in the chilling (7 ^{o}C day / 5 ^{o}C night) cabinet for a further 21 days. Five days after being returned to growth temperatures, *P. vulgaris* plants that were exposed to 9 days of chilling appeared to have been set back in their vegetative development when compared to *P. vulgaris* plants that had not experienced the chilling cabinet (Figure 3.3). Nine-day chilled *P. vulgaris* plants took 10% longer time to flower than did non-chilled ones (46 vs 42 days). Nine-day chilled *P. angustissimus* plants took 26% longer time to flower (68 vs 54 days). Figure 3.4 shows *P. angustissimus* plants five days after being returned to growth temperatures after exposure to 9 days of chilling (7 ^{o}C day / 5 ^{o}C night) compared to *P. angustissimus* plants that were not chilled. After 42 days of chilling (7 ^{o}C day / 5 ^{o}C night), *P. vulgaris* plants appeared to have grown relatively more than *P. angustissimus* plants. New trifolate leaves were a yellow-green colour, while older trifolate leaves developed patches of purple-brown colour (Figure 3.5).

Table 3.7. Effect of chilling in *P. vulgaris* and *P. angustissimus* on chlorophyll a/b ratio.

	***P. vulgaris* average chlorophyll a/b ratio (cm±SD)**		***P. angustissimus* average chlorophyll a/b ratio (cm±SD)**	
	23/18 °C	**7/5 °C**	**23/18 °C**	**7/5 °C**
Day 1	1.3 (0.3)	1.1 (0.3)	1.2 (0.3)	0.9 (0.1)
Day 2	1.5 (0.3)	1.7 (0.4)	1.6 (0.2)	1.4 (0.5)
Day 3	0.9 (0.7)	3.0 (0.5)	1.3 (0.3)	1.4 (0.4)
Day 4	1.2 (0.3)	1.7 (0.3)	1.1 (0.3)	0.9 (0.1)
Day 5	0.9 (0.3)	1.1 (0.5)	1.2 (0.5)	1.1 (0.4)

All plants were grown for 21 days in 23 °C day / 18 °C night temperatures. A subset of each species was transferred to a chamber set to 7 °C day / 5 °C night. Each day after transfer, chlorophyll a/b ratio was calculated for four warm and four chilled plants of each species. Means and standard deviations of ratios are presented.

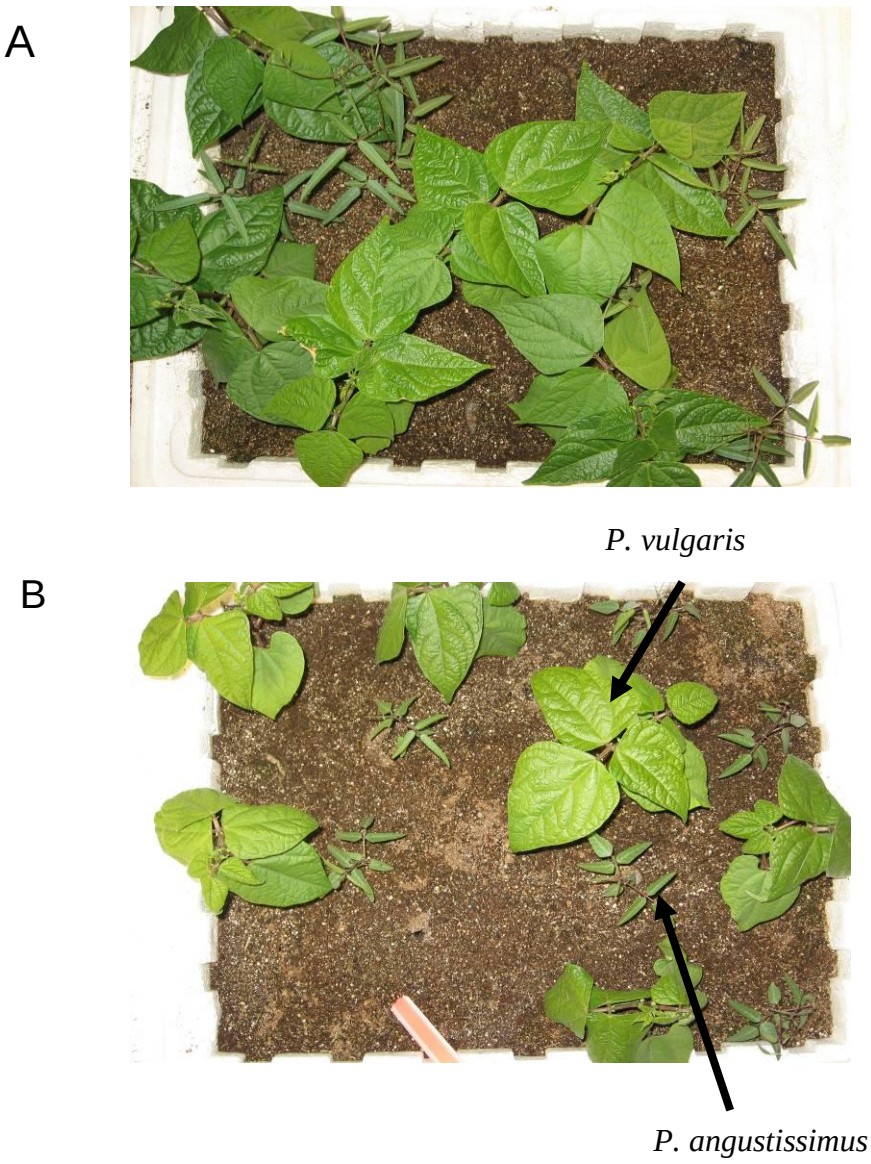

Figure 3.2. Photographic comparison of non-chilled and three day-chilled *Phaseolus* plants.

All plants were grown for 21 days in 23 ^{o}C day / 18 ^{o}C night temperatures. A subset of each species was transferred to a chamber set at 7 ^{o}C day / 5 ^{o}C night. (*A*) Plants grown at 23 ^{o}C day / 18 ^{o}C night for 24 days. (*B*) Plants grown under same conditions for 21 days followed by three days at 7 ^{o}C day / 5 ^{o}C night.

Figure 3.3. Photographic comparison of nine day chilled and non-chilled *P. vulgaris* plants.

All plants were grown for 21 days in 23 ^{o}C day / 18 ^{o}C night temperatures. A subset of each species was transferred to a chamber set at 7 ^{o}C day / 5 ^{o}C night. (Left) Nine day chilled *P. vulgaris* plants five days after return to growth temperatures. (Right) Non-chilled *P. vulgaris* plants of the same age.

Figure 3.4. Photographic comparison of nine day chilled and non-chilled *P. angustissimus* plants.

All plants were grown for 21 days in 23 ^{o}C day / 18 ^{o}C night temperatures. A subset of each species was transferred to a chamber set at 7 ^{o}C day / 5 ^{o}C night. (Right) Nine day chilled *P. angustissimus* plants five days after return to growth temperatures. (Left) Non-chilled *P. angustissimus* plants at the same age.

Figure 3.5. Photographic comparison of 42 day chilled *P. vulgaris* and *P. angustissimus* plants.

(Right) *P. angustissimus* and (Left) *P. vulgaris* plants that were chilled at 7 ^{o}C day / 5 ^{o}C night for 42 days. Plants were grown for 21 days in 23 ^{o}C day / 18 ^{o}C night temperatures prior to chilling.

3.4 Discussion

These studies on freezing tolerance demonstrate that treating *P. vulgaris* and *P. angustissimus* to low temperatures (7 ^{o}C day / 5 ^{o}C night) for 72 hours did not result in large contrasts between the two species with regard to freezing tolerance (Table 3.1). Neither species responded to these cold acclimation conditions with levels of freezing tolerance seen in other species, such as *Arabidopsis* or *M. sativa*. Xin and Browse (1998) decreased the LT50 (temperature where 50% of plants are killed) of *Arabidopsis* from -6.0 ^{o}C to -14 ^{o}C after two days at 4 ^{o}C, whereas the hardy legume *M. sativa* achieved maximal freezing tolerance of -16 ^{o}C after two weeks at 2.0 ^{o}C followed by another two weeks at -2.0 ^{o}C night (Monroy et al., 1993). Optimal cold acclimation treatments range considerably across plant species. Thus, an increase in freezing tolerance in these *Phaseolus* species may be achieved by shifting the duration of cold acclimation regimes. However, Balasubramanian (2002) found that 7^{o}C day / 5^{o}C night temperatures for nine days did not drastically alter the LT50 for many *P. vulgaris* cultivars or for *P. angustissimus*.

Why do some plant species increase their freezing tolerance in response to cold acclimation treatments while others do not? For example, the LT50 of non-acclimated *Arabidopsis* is typically -3 ^{o}C, but after acclimation the LT50 was -10 ^{o}C (Gilmour et al., 1988). Similar increases in freezing tolerance after cold acclimation treatments were observed in spinach (Guy et al., 1985), which increases in freezing tolerance from -6 ^{o}C to -10 ^{o}C; in potato, for which the LT50 decreases from -5 ^{o}C to -11.5 ^{o}C (Lee et al., 1992); and in rye (Webb et al., 1994), which increases in freezing tolerance from -6 ^{o}C to -21 ^{o}C. Tomato, on the other hand, is generally not considered to increase its

freezing tolerance level in response to cold acclimation. The LT50 of tomato is -2.0 ^{o}C with or without cold acclimation treatment (Zhang et al., 2004). Among legumes, alfalfa has the greatest freezing tolerance even without cold acclimation treatment (-7 ^{o}C; Meyer and Badaruddin, 2001), but after 2 weeks of cold acclimation treatment its freezing tolerance becomes −16 ^{o}C (Monroy et al., 1993). Thus, according to the present study, both *P. vulgaris* and *P. angustissimus* would rank among the least responsive to cold acclimation treatment among plants regardless of cold acclimation treatments. It is likely that the divergence in cold acclimation responses and freezing tolerance is a result of different selection pressures that have shaped the genomes of these plants over time. As such, a functional genomics approach to understanding low temperature response in *P. vulgaris* and *P. angustissimus* is essential to obtain a greater understanding of these responses of these species to low temperature treatment.

Both *P. vulgaris* and *P. angustissimus* plants exhibited reduced growth rates (Table 3.2) upon chilling treatment and did not resume normal growth under these conditions over the course of this experiment, which is consistent with many chilling sensitive plants. Nine day chilled plants of both species showed little sign of permanent chilling injury after being returned to normal growth temperatures for 14 days, other than they appeared smaller than their age-matched non-chilled controls (Figure 3.3, 3.4). However, nine-day chilled *P. vulgaris* achieved flowering stage relatively quicker than similarly chilled *P. angustissimus*. Under these conditions, *P. angustissimus* showed recovery from chilling consistent with results seen in maize seedlings, which required four days to recover normal leaf expansion rates after being treated to 24 hours of 0.3 ^{o}C (Creencia and Bramlage, 1971). It is not known whether rapid recovery rates,

as measured by days to flowering, are indicative of improved chilling tolerance in plants, but for cultivation purposes, it is important for growers to consider that a cultivar which can rapidly recover from a prolonged chilling event is more likely to reach maturity before fall frost occurs.

In observing Table 3.2, it is curious to see slight decreases in average plant height in both species after transfer to chilling conditions. In this regard, it is important to note the slight variations in height on plant-to-plant basis likely accounts for the apparent 'shrinkage' of these plants, and that plants transferred to the chilling cabinet already demonstrated variations in plant height. Thus, the decrease in plant height observed in these chilled plants is likely due to chance selection of smaller plants, not an actual phenomenon of 'shrinkage', and that more experimental replications with greater numbers of plants would likely have revealed no change in height after exposure to chilling temperatures.

With regard to tissue desiccation, the rapid dehydration of chilled *P. vulgaris* plants in this experiment was consistent with results seen by Vernieri et al. (2001). In contrast, *P. angustissimus* plants were not perturbed in a similar manner, maintaining tissue water content values consistent with their non-chilled controls (Table 3.5). Taken together, these results indicate that a potential key to understanding the ability of the wild species to avoid chilling induced desiccation may lay in the ability of *P. vulgaris* to maintain internal water content. One hypothesis that can be made regarding desiccation response is that since the leaf size and composition of these two species is noticeably different that a reason for the cultivated species to lose more water is because it has a

relatively greater potential to lose water from its larger and more succulent leaves compared to the wild species *P. angustissimus*.

Vernieri (2001) found that *P. vulgaris* (cv. Borlotto Nano) suffered severe water stress upon chilling temperature incubation, as leaf water potential, osmotic potential, and turgor pressure declined rapidly, because the roots did not properly adjust to the water demands of the aerial tissues. Research indicates that this disconnect in the root-shoot communication system may be mediated by abscisic acid (ABA), as chilling sensitive mung bean (*Vigna radiata* L.) recovered after five days of root chilling soon after an increase in endogenous ABA in both leaf and root tissues (Bagnall et al., 1983). Exogenous applications of ABA increased water conductance in chilled sunflower (Ludewig et al., 1988) and soybean (Markhart, 1984). An interesting hypothesis would be that *P. angustissimus* better adjusts to chilling temperatures because of its ABA-mediated root-shoot communication mechanisms.

Another mechanism by which plants maintain internal water potential is to limit transpirational water loss via stomatal closure. Leaf dehydration in chilling conditions can be averted by rapid stomatal closure (Davies et al., 1982). Chilling tolerant plant species, including *Ulmus americana* L. and *Fraxinus pennsylvanica* L., display stomatal closure in chilling conditions (Kozlowski and Pallardy, 1979), while chilling sensitive species, including *P. vulgaris* (Wilson, 1976) and *O. sativa* (Lee et al., 1993) display open stomates under chilling conditions. During their investigations as to the mechanisms of low temperature induced stomatal closure, Wilkinson et al. (2001) found stomatal closure in chilling tolerant *Commelina communis* L. was linked to guard cell sensitivity to apoplastic calcium level, ruling out other factors such as ABA signaling,

temperature effects on guard cells, and photosynthetic responses to low temperature. In the same experiments, Wilkinson et al. (2001) found that chilling sensitive *N. tabacum* plants were relatively insensitive to apoplastic calcium and did not close stomatal guard cells under low temperature conditions.

The low temperature conditions used in this experiment were as severe as the natural temperature fluctuations that these plants would endure at this stage in their life-cycle in the field. While overnight lows of around 2-5 ^{o}C are common in the months of May and June in Saskatoon, Saskatchewan, daytime high temperatures normally far exceed the 7 ^{o}C highs of the chilling cabinet, thereby giving plants a natural reprieve from chilling temperatures during the high light intensities experienced during daytime.

The decrease of tissue water content (Levitt, 1980) and decrease in chlorophylls (Walker et al., 1991) as a result of exposure to chilling temperatures observed in this experiment (Table 3.5) are well known phenomena in chilling-sensitive plants. Altered chlorophyll fluorescence patterns observed in the chilled leaves in this study is indicative of early symptoms of chilling stress, which indicates possible damage to the photosynthetic apparatus, potentially resulting in reduced CO_2 fixation (Walker et al., 1991). Injury from photo-oxidation and reduced photosynthetic efficiency is among the most immediate effects of chilling stress. The negative effect of cold treatment on photosynthetic capacity and chlorophyll content may be due to alterations in lipid and protein components of thylakoid membranes (Mostowska, 1997). For instance, exposing pea (*Pisum sativum* L.) to 5 ^{o}C resulted in ultra-structural damage to the inner membrane of chloroplasts (Wise and Naylor, 1987b). Chloroplast damage as a result of chilling injury will result in loss of chlorophyll, and reactive oxygen species will

damage the lipids of cell membranes causing leakage of cell contents and loss of water (Koscielnak, 1993; Wilson, 1976). Therefore, visual symptoms of chilling damage include yellowing and inhibition plant growth (Salveit and Morris, 1990).

This study reports on experiments undertaken to investigate how two species of bean, *Phaseolus vulgaris* and *Phaseolus angustissimus*, cope with chilling temperatures. In particular, it was shown that neither species demonstrated a great capacity, compared to *Arabidopsis* and *M. sativa*, for instance, for cold acclimation under these experimental conditions, as plants of each species still demonstrated LT50 levels above -3.0 ^{o}C. It was also shown that while both bean species halted normal growth and had decreased chlorophyll content when incubated at chilling temperatures, *P. angustissimus* was able to maintain aerial tissue water content under chilling temperatures, whereas *P. vulgaris* suffered a depletion of water content over the first three days of chilling.

4. Identification and characterization of genes related to low temperature exposure in *Phaseolus* species using interspecific macroarray hybridizations

4.1 Introduction

The study of gene function in the context of cellular physiology and biochemistry presents a major goal in the study of plant biology. Genes can be identified first by their nucleic acid sequence and their roles in cellular processes can be then further understood by their expression under various conditions. One of the most common tools used to carry out this type of analysis is profiling of large scale cDNA sets using array technology.

Gene expression profiling enables researchers to study alterations in cellular processes in great detail, including studies on development (Fernandes et al., 2002), metabolism (White et al., 2000), and responses to biotic and abiotic stresses (Kawasaki et al., 2001; Chen et al., 2002). Genes that are found to be responsive in abiotic stress conditions have been shown to generate specific proteins and metabolites that are crucial in protecting cells and tissues. Furthermore, genes found to be stress responsive lead to a transduction of signals that arise from the response to stress (Hwang et al., 2005). In a comprehensive study of *Arabidopsis* transcriptomic responses to low temperature stress, Seki et al. (2001) found that proper adaptation to low temperatures requires highly coordinated shifts in gene expression. Additionally, Shinozaki and

Yamaguchi-Shinozaki (2000) detailed significant cross-talk (interactions of gene signaling networks) across abiotic stresses of low temperature and drought, while Rabbani et al. (2003) found that similar cross-talk occurs between these abiotic stresses, high-salinity stress, and abscisic acid application. In a microarray study using full-length cDNAs of *Arabidopsis*, Narusaka et al. (2004) found widespread cross-talk between biotic and abiotic stresses, particularly through modulation of cytochrome P450 expression. Thus, use of array technology enables researchers to unveil cellular and molecular mechanisms and plant processes in great detail.

Plants vary greatly in their ability to tolerate cold temperatures (Sakai and Larcher, 1987). Many species from tropical regions, such as tomato, maize and rice, are unable to tolerate freezing and suffer chilling injury when exposed to temperatures in the range of 0 to 12^{o}C. In contrast, plants from temperate regions, such as wheat, canola and *Arabidopsis*, are able tolerate both chilling and freezing temperatures. A major limitation to the production of common bean (*Phaseolus vulgaris* L.) in arable areas of Saskatchewan is that episodic and prolonged chilling trends are common at the beginning of the growing season (Balasubramanian et al., 2004; Miller et al., 2002). In previous studies, Balasubramanian found that *Phaseolus angustissimus* L., a wild bean species, displays greater freezing tolerance than the cultivated *P. vulgaris*, and in Chapter 2, it is shown that *P. angustissimus* responds better to low temperature exposure than *P. vulgaris*. However, there is very little information on the molecular aspects of chilling responses in these bean species, and the genes involved in chilling tolerance have not been identified in these species.

Lack of genomic resources has made the study of the functional genomics of low temperature stress in *Phaseolus* species difficult. However, other legumes such as alfalfa (*Medicago sativa* L.) have more developed genomic tools which include cDNA libraries. Recent successes in cross-species cDNA hybridization studies with *Arabidopsis* using *Arabidopsis halleri*, *Brassica napus*, *Thlaspi arvense*, and *Thellungiella halophila*, have opened new avenues for studying genomics in resource-poor species (Becher et al. 2004; Lee et al., 2004; Taji et al., 2002; Gong et al., 2005; Sharma et al., 2007).

In this study, 1672 clones of a cDNA library derived from cold acclimated alfalfa were used to hybridize cDNAs derived from non-chilled and three-day chilled *P. vulgaris* and *P. angustissimus*. These efforts represents one of the first studies to use interspecific cDNA hybrizations to examine low temperature stress of the *Phaseolus* genus, and results of this research give insight into the transcriptional responses of these species to cold stress. Due to the limited genomic resources available in *Phaseolus*, a direct study of low temperature response in bean using genomic tools could not be readily conducted. Thus, in order to obtain a tentative understanding of transcriptional responses in these *Phaseolus* species, an interspecific macroarray experiment was conducted using cDNAs derived from cold acclimated *M. sativa* crowns. In the chilling sensitive *P. vulgaris*, 453 of these clones (77 up-regulated and 376 down-regulated) were shown to be chilling-inducible, whereas 199 clones (134 up-regulated and 65 down-regulated) were responsive in chilled *P. angustissimus*. The molecular characteristics of these clones and the possible implications for *Phaseolus* are discussed.

4.2 Materials and methods

4.2.1 Plant materials and growth conditions

Seeds of cultivated common bean (*Phaseolus vulgaris* L. cv ICA Pijao) and wild bean (*P. angustissimus* L. PI535272) were germinated and grown for 21-days as described in 3.2.1. Plants selected for chilling were transferred to a growth chamber, preset to 7^{o}C day / 5^{o}C night with the same light conditions as the original growth chamber, for 72 hours. Aerial tissues of non-chilled plants and of plants chilled for 72 hours were immediately frozen in liquid nitrogen and stored at -80^{o}C until needed. For a second biological replicate, this process was repeated. All plants were sampled at subjective nightfall.

4.2.2 RNA preparation

RNA was extracted from non-chilled and chilled samples using Concert Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. All RNA samples were treated with RNAse-free DNAse I and cleaned using the E.Z.N.A. MicroElute RNA Clean-up Kit (Omega Biotech, Doraville, GA, USA) according to manufacturer's instructions. An mRNA purification kit (Amersham Biosciences, Piscataway, NJ, USA) was used to purify mRNA from these RNA samples according to manufacturer's instructions.

4.2.3 Macroarray preparation

PCR products were derived from cloning vector pCR 2.1 (Invitrogen, Carlsbad, California, USA) using M13 forward primer (5' – CATTTTGCTGCCGGTC -3') and M13 reverse primer (5' – CAGGAAACAGCTATGAC – 3'). *Medicago sativa* cDNA inserts were selected from a cDNA library constructed by Dr. Serge Laberge and his

research team at Agriculture and Agri-Food Canada (Ste-Foy) and used for printing the array. A total of 1672 *M. sativa* cDNAs were selected from this library to print on the array. Of these cDNAs, 336 did not have sufficient sequence data to conduct BLAST searches. Gene Ontology tables of the cellular component, molecular function, and biological processes for the *M. sativa* cDNAs selected for the array and that had sufficient sequence data are shown in Table 4.1. A subset of 40 cDNAs were specifically selected on the basis of their known function or putative function that may be linked to stress response in other species.

The cDNAs that were randomly selected for this experiment were not filtered to insure uniqueness. The *M. sativa* cDNAs were derived from transcripts of *M. sativa* crowns that were treated to cold acclimation temperatures (two weeks at 2 ^{o}C day / -2 ^{o}C night). Each *M. sativa* cDNA PCR product that was printed on the array is referred to as a 'spot'. In the first experiment, PCR products were printed individually on a positively charged 7.5- 11.5 cm Hybond membrane (Appligen-Oncor, Illkirch, France) using a Beckman 96-pin high-density replicating tool (Biomek, Fullerton, California, USA). In the second experiment, PCR products were deposited on Hybond membranes using a robospotter (BioGrid, BioRobotics, United Kingdom). After spotting was completed, all membranes were denatured in 1.5 M NaCl - 0.5 M NaOH and neutralized in 1.5 M NaCl - 0.5 M Tris-HCl (pH 7.5)-1 mM EDTA. DNA fragments were then cross-linked to the membrane with 120 mJ of ultraviolet light using a Stratalinker UV transilluminator (Stratagene, La Jolla, CA, USA.).

Table 4.1. Gene ontology (GO) analysis of cellular component, molecular function, and biological processes of *M. sativa* clones on the array.

GO Cellular Component	% of total annotations	GO Molecular Function	% of total annotations	GO Biological Process	% of total annotations
cell wall	0.8	**DNA or RNA binding**	6.7	**cell organization and biogenesis**	5.3
chloroplast	7.4	**hydrolase activity**	9.5	**developmental processes**	3.4
cytosol	4.8	**kinase activity**	5.0	**DNA or RNA metabolism**	0.3
ER	0.8	**nucleic acid binding**	2.6	**electron transport or energy pathways**	2.1
extracellular	0.6	**nucleotide binding**	3.7	**other biological processes**	3.8
Golgi apparatus	0.6	**other binding**	9.7	**other cellular processes**	22.0
mitochondria	4.7	**other enzyme activity**	11.0	**other metabolic processes**	22.0
nucleus	9.0	**other molecular functions**	1.8	**protein metabolism**	8.6
other cellular components	0.3	**protein binding**	8.4	**response to abiotic or biotic stimulus**	6.3
other cytoplasmic components	11.8	**receptor binding or activity**	0.6	**response to stress**	5.8
other intracellular components	15.8	**structural molecule activity**	5.7	**signal transduction**	2.1
other membranes	16.8	**transcription factor activity**	4.8	**transcription**	3.0
plasma membrane	1.0	**transferase activity**	7.3	**transport**	4.0
plastid	4.0	**transporter activity**	6.8	**unknown biological processes**	11.2
ribosome	6.8	**unknown molecular functions**	16.3		
unknown cellular components	14.6				

4.2.4 cDNA labeling and macroarray hybridization

A total of 2 µg of mRNA derived from samples from each species, biological replicate, and temperature regime were reverse transcribed separately to cDNA using the Promega cDNA Synthesis System (Promega, Madison, WI, USA) according to manufacturer's instructions. These cDNAs were radiolabelled with ^{33}P dCTP using the Rediprime cDNA Labeling System (Amersham Biosciences, Piscataway, USA) according to the manufacturer's instructions. Radiolabelleled cDNAs were placed in 7 mL of hybridization solution (2x SSC, 0.5% SDS, and 0.25% milk powder) and allowed to hybridize to membranes at 55 °C overnight. This hybridization was followed by three 45-minute washes (2x SSC, 0.5% SDS) and three further washes (2x SSC, 0.1% SDS) of 20 min each. Hybridized membranes were exposed overnight to a phosphoimaging screen (Kodak, Rochester, New York, USA). Screens were scanned using (BioRad Molecular Imager FX, BioRad Laboratories, Hercules, California, USA) and images were stored digitally.

4.2.5 Macroarray analysis

ArrayGuage version 2.1 (Fuji-film; Tokyo, Japan) was used for image analysis. Since the second biological replicate included spot replications, an average intensity was calculated for each clone such that each normalized spot intensity value was expressed as percent of average spot intensity. The intensity for each spot was calculated as a value above local membrane background. These individual spot values were calculated in each of the two biological replicates, and average spot intensities

were pooled between the two biological replicates for each spot. Ratios of pooled average spot intensity were calculated for each species in each condition. Genes were considered chilling responsive if the spot intensity on the membrane treated with cDNAs derived from 72 hour chilled (7 ^{o}C day / 5 ^{o}C night) plants were at least three times greater or less than that of the intensity of the corresponding spot on the membrane treated with cDNAs derived from non-chilled plants once normalized for background. This threshold was considered appropriate for highlighting large changes in gene expression levels while enabling the analysis of a suitable number of genes in both bean species. *M. sativa* sequences corresponding to chilling responsive genes were analyzed using Basic Local Alignment Search Tool (BLAST; Altschul et al., 1997) bioinformatic software, with best BLAST scores used to identify putative orthologues. Gene Ontology (GO) annotations were performed on best BLAST hits using GO-Slim bioinformatic software (http://www.*Arabidopsis* .org/tools/bulk/go/index.jsp).

4.3 Results

4.3.1 Up- and down-regulated genes in chilled *P. vulgaris* and *P. angustissimus*

P. vulgaris showed a greater number of transcriptionally up- or down-regulated genes than *P. angustissimus*. In the first biological replicate, *P. vulgaris* had 338 transcriptionally altered genes (137 up- and 201 down-regulated), while *P. angustissimus* had 70 genes transcriptionally altered (37 up- and 33 down-regulated). In the second biological replicate, *P. vulgaris* transcriptionally altered 762 genes (102 up- and 660 down-regulated), while *P. angustissimus* transcriptionally altered 551 genes (338 up- and 213 down-regulated).

P. vulgaris showed a trend towards down-regulation of responsive cDNAs compared to *P. angustissimus*. Fifty-nine percent of *P. vulgaris* transcriptionally responsive genes were found to be down-regulated in the first replicate and 87% in the second replicate, while *P. angustissimus* showed a trend of transcriptional up-regulation, as 47% of *P. angustissimus* transcriptionally responsive genes were found to be down-regulated in the first biological replicate and 39% in the second replicate.

P. vulgaris genes with consistent (up-or down-regulation in each biological replicate of the array) expression and known function based on BLAST analysis, are shown in Table 4.2. Across the two biological replicates, *P. vulgaris* showed consistent transcriptional regulation of 176 genes, with 15 genes consistently up-regulated and 161 genes consistently down-regulated. Comparing the first biological replicate to the second biological replicate in *P. vulgaris*, 125 genes switched their expression pattern from up-regulated to down-regulated and 65 genes switched their expression pattern from down-regulated to up-regulated, while 40 genes did not express consistent up-regulation and 78 genes did not express consistent down-regulation. Comparing the second biological replicate to the first biological replicate in *P. vulgaris*, 128 genes switched their expression pattern from up-regulated to down-regulated and 252 genes switched their expression pattern from down-regulated to up-regulated, while 37 genes did not express consistent up-regulation and 381 genes did not express consistent down-regulation.

P. angustissimus genes with consistent (up- or down-regulation in each biological replicate of the array) expression and known function based on BLAST analysis are shown in Table 4.3. Across the two biological replicates, *P. angustissimus*

showed consistent transcriptional regulation of 24 genes, with 16 genes consistently up-regulated and eight genes consistently down-regulated. Comparing the first biological replicate to the second biological replicate in *P. angustissimus*, 31 genes switched their expression pattern from up-regulated to down-regulated and 60 genes switched their expression pattern from down-regulated to up-regulated, while 18 genes did not express consistent up-regulation and 31 genes did not express consistent down-regulation. Comparing the second biological replicate to the first biological replicate in *P. angustissimus*, 260 genes switched their expression pattern from up-regulated to down-regulated and 123 genes switched their expression pattern from down-regulated to up-regulated, while 163 genes did not express consistent up-regulation and 185 genes did not express consistent down-regulation.

Averaging the normalized intensity values of each gene enabled the results from the two biological replicates to be pooled. Figure 4.1 shows plotting results for each gene as a ratio of control and chilled values of normalized pooled specific spot hybridization intensity to normalized average total spot hybridization intensity across the two biological replicates as a means of comparing the responses in gene expression. In Figure 4.1, *P. vulgaris* is shown to have a greater overall response in gene expression compared to *P. angustissimus*, as comparatively larger numbers of gene expression values to deviate greatly from a 1:1 ratio after three days of chilling in the plot of signal level for each spot for controlled vs. chilled plants. Figure 4.1 also demonstrates how most of the genes sampled using *P. vulgaris* showed down-regulation of gene expression. *P. angustissimus*, on the other hand, does not show as strong an overall

Table 4.2 Effect of low temperature exposure on expression levels of *P. vulgaris* genes in aerial tissue as revealed by hybridization to a macroarray of *M. sativa* cDNA clones.

M. sativa clone ID	Best BLAST hit	ratio of expression level (7 °C/5 °C)/(23 °C /18 °C) replicate 1	replicate 2
Up-regulated			
Ms01_01e05	zinc finger (C3HC4-type RING finger) family protein [*Arabidopsis thaliana*]	4.26	8.99
Ms03_22a01	senescence-associated protein [*Pisum sativum*]	7.24	3.93
Ms03_26c09	enolase [*Musa acuminata*]	3.44	3.34
Ms03_27c03	membrane channel protein [*Medicago sativa*]	4.31	7.41
Ms03_28a12	protein [*Oryza sativa* (indica cultivar-group)]	19.68	9.22
Ms03_28c09	environmental stress-induced protein [*Medicago sativa*]	16.80	3.24
Ms03_29h05	abscisic acid activated protein [*Medicago sativa*]	8.48	3.24
Ms03_30a01	probable ATP-dependent RNA helicase [imported] [*Arabidopsis thaliana*]	24.11	188.79
Ms03_33e12	mRNA for prolin-rich protein (PvPRP1) [*P. vulgaris*]	4.16	10.04
Down-regulated			
Ms01_01d01	protein GRO10 [*Euphorbia esula*]	0.14	0.20
Ms01_02a03	cytochrome P450 [*Arabidopsis thaliana*]	0.05	0.13
Ms01_02b03	initiation factor 4, eIF4-like protein [*Arabidopsis thaliana*]	0.08	0.23
Ms01_02c01	methionine synthase [*Glycine max*]	0.10	0.03
Ms01_02c02	peroxidase prx11 precursor – spinach	0.22	0.31
Ms01_02f08	delta-COP [*Zea mays*]	0.08	0.07
Ms01_02g04	anthocyanidine rhamnosyl-transferase [*Capsicum annuum*]	0.23	0.14
Ms01_02h08	RNA helicase RH23 [*Arabidopsis thaliana*]	0.10	0.03
Ms01_03a08	proteasome non-ATPase regulatory subunit 7 [*Arabidopsis thaliana*]	0.21	0.09
Ms01_03a09	ribosomal protein [*Oryza sativa* (japonica cultivar-group)]	0.11	0.23
Ms01_03b06	Caffeoyl-CoA O-methyltransferase 2 [*Eucalyptus globulus*]	0.13	0.26
Ms01_03b11	P450, putative [*Arabidopsis thaliana*]	0.19	0.15
Ms01_03c07*	Beta-amylase (1,4-alpha-D-glucan maltohydrolase) [*Medicago sativa*]	0.15	0.28
Ms01_03e02	dehalogenase-like hydrolase family protein [*Arabidopsis thaliana*]	0.21	0.29
Ms01_03e07	Expansin-related protein 1 precursor (At-EXPR1) [*Arabidopsis thaliana*]	0.07	0.20
Ms01_03g12	senescence-associated protein [*Pisum sativum*]	0.12	0.15
Ms01_03h08	synthetase [*Triticum aestivum*]	0.26	0.06
Ms01_04d03	kinase-like protein [*Oryza sativa* (japonica cultivar-group)]	0.22	0.24
Ms01_05a02	DnaJ protein homolog [*Phaseolus vulgaris*]	0.11	0.17
Ms01_05b09	ribosomal protein S27 [*Arabidopsis thaliana*]	0.14	0.05
Ms01_05c02	EF hand family protein [*Arabidopsis thaliana*]	0.32	0.30
Ms01_05c09	brassinosteroid receptor [*Pisum sativum*]	0.31	0.05
Ms01_05d07	probable lectin 2 precursor [*Medicago sativa*]	0.16	0.07
Ms01_05d08	dehydrin-related protein [*Pisum sativum*]	0.23	0.23
Ms01_05e04	AP2/EREBP transcription factor [*Arabidopsis thaliana*]	0.21	0.06
Ms01_05e07	histidyl-tRNA synthetase [*Triticum aestivum*]	0.27	0.03

Ms01_05f04	A beta-lactamase TEM-87 [*Proteus mirabilis*]	0.29	0.14
Ms01_05g03	probable sterol 24-C-methyltransferase [*Glycine max*]	0.22	0.08
Ms03_19f05	protein kinase 4 (CIPK4) [*Arabidopsis thaliana*]	0.30	0.08
Ms03_19f10*	polygalacturonase inhibitor protein [*Brassica napus*]	0.24	0.08
Ms03_19h08	zinc finger domain-containing protein (ADOF1) [*Arabidopsis thaliana*]	0.20	0.14
Ms03_19h10	Alcohol dehydrogenase 1 [*Pisum sativum*]	0.23	0.15
Ms03_20g09*	Beta-amylase (1,4-alpha-D-glucan maltohydrolase) [*Medicago sativa*]	0.25	0.03
Ms03_20h05*	dehydrin-like protein [*Medicago sativa*]	0.23	0.01
Ms03_21e03	erythrocyte surface antigen [*Plasmodium yoelii yoelii*]	0.31	0.12
Ms03_21h07	track-binding protein homologue [*Cicer arietinum*]	0.17	0.25
Ms03_22b02*	dehydrin-like protein, CAS18 [*Medicago sativa*]	0.24	0.10
Ms03_22e09	omega-3 fatty acid desaturase [*Glycine max*]	0.25	0.08
Ms03_22e11	RNA recognition motif (RRM)-containing protein [*Oryza sativa*]	0.24	0.01
Ms03_23d02	glycogen (starch) synthase [*Astragalus membranaceus*]	0.30	0.29
Ms03_23h11*	dehydrin-like protein, CAS18 [*Medicago sativa*]	0.20	0.30
Ms03_24b02	phi-1 [*Nicotiana tabacum*]	0.25	0.03
Ms03_24e10	ribosomal protein L13A (RPL13aD) [*Arabidopsis thaliana*]	0.10	0.14
Ms03_24e11*	dehydrin-like protein [*Medicago sativa*]	0.21	0.10
Ms03_25c01*	Alcohol dehydrogenase 1 [*Pisum sativum*]	0.15	0.17
Ms03_25c03	KE2 family protein [*Arabidopsis thaliana*]	0.15	0.07
Ms03_25c09	ribosomal protein S21 (RPS21C) [*Arabidopsis thaliana*]	0.31	0.02
Ms03_25d01	kinase protein [*Cicer arietinum*]	0.17	0.14
Ms03_25d12	kinase [*Lycopersicon esculentum*]	0.27	0.02
Ms03_25f12	eukaryotic initiation factor 4 [*Cryptosporidium parvum*]	0.28	0.25
Ms03_25h06	protein 1 / CIP1 [*Arabidopsis thaliana*]	0.19	0.26
Ms03_26e10*	dehydrin-like protein, CAS18 [*Medicago sativa*]	0.18	0.11
Ms03_26e12	gamma-aminobutyric acid (GABA-A) receptor, subunit epsilon	0.24	0.08
Ms03_26h07	NAC1 [*Medicago truncatula*]	0.14	0.05
Ms03_27a01	Pyruvate decarboxylase isozyme 1 (PDC) [*Pisum sativum*]	0.31	0.12
Ms03_27e11	Ole e 1 allergen and extensin family protein [*Arabidopsis thaliana*]	0.20	0.22
Ms03_27h12	Pyruvate decarboxylase isozyme 1 (PDC) [*Pisum sativum*]	0.14	0.07
Ms03_28c04	dormancy-associated protein [*Pisum sativum*]	0.18	0.16
Ms03_28d04	RNA polymerase [Oyster mushroom isometric virus II]	0.11	0.08
Ms03_28g01	division cycle protein 48, putative / CDC48, putative [*Arabidopsis thaliana*]	0.26	0.07
Ms03_29a09	DEAD box protein [*Pisum sativum*]	0.19	0.06
Ms03_29c01*	dehydrin-like protein [*Medicago sativa*]	0.09	0.10
Ms03_29h08	kinase 2 (ADK2) [*Arabidopsis thaliana*]	0.29	0.14
Ms03_30b03	AAFC_ECORC_cold_stressed [*Descurainia sophia*]	0.11	0.13
Ms03_30c08*	dehydrin-like protein [*Medicago sativa*]	0.19	0.13
Ms03_30c10	kinase family protein [*Arabidopsis thaliana*]	0.16	0.32
Ms03_30e01	domain-containing protein / GAT domain-containing protein [*Arabidopsis thaliana*]	0.17	0.04
Ms03_30e12	remorin family protein [*Arabidopsis* thaliana]	0.07	0.09
Ms03_30f02	epimerase/dehydratase family protein [*Arabidopsis thaliana*]	0.29	0.02
Ms03_30f10	synthetase-like protein [*Arabidopsis* thaliana]	0.29	0.05

- * genes previously associated with response to low temperatures.
- Only genes with known identity based on a BLAST search and consistent expression across two biological replicates are shown.

Table 4.3 Effect of low temperature exposure on expression levels of *P. angustissimus* genes in aerial tissue as revealed by hybridization to a macroarray of *M. sativa* cDNA clones.

M. sativa clone ID	Best BLAST hit	ratio of expression level (7 °C/5 °C)/(23 °C /18 °C) replicate 1	replicate 2
Up-regulated			
Ms01_02a03	cytochrome P450 [*Arabidopsis thaliana*]	5.29	3.18
Ms01_03b11	P450, putative [*Arabidopsis thaliana*]	4.10	12.22
Ms01_04h02	helix-loop-helix-like protein [*Cucumis melo*]	5.71	5.04
Ms03_23e08	senescence-associated protein [*Pisum sativum*]	5.01	4.53
Ms03_29b03	ribosomal protein S9 (RPS9C) [*Arabidopsis thaliana*]	3.55	3.58
Ms03_29b09	Thiamine pyrophosphate (TPP) family [*Arabidopsis thaliana*]	17.10	3.44
Ms03_30c10	kinase family protein [*Arabidopsis thaliana*]	3.73	5.31
Down-regulated			
Ms03_21e03	proline-rich extensin-like protein [*Arabidopsis thaliana*]	0.11	0.11
Ms03_22b02*	dehydrin-like protein, CAS18 [*Medicago sativa*]	0.21	0.12
Ms03_25h06	protein 1 / CIP1 [*Arabidopsis thaliana*]	0.22	0.06

- * genes previously associated with response to low temperatures
- Only genes with known identity based on a BLAST search and consistent expression across two biological replicates are shown.

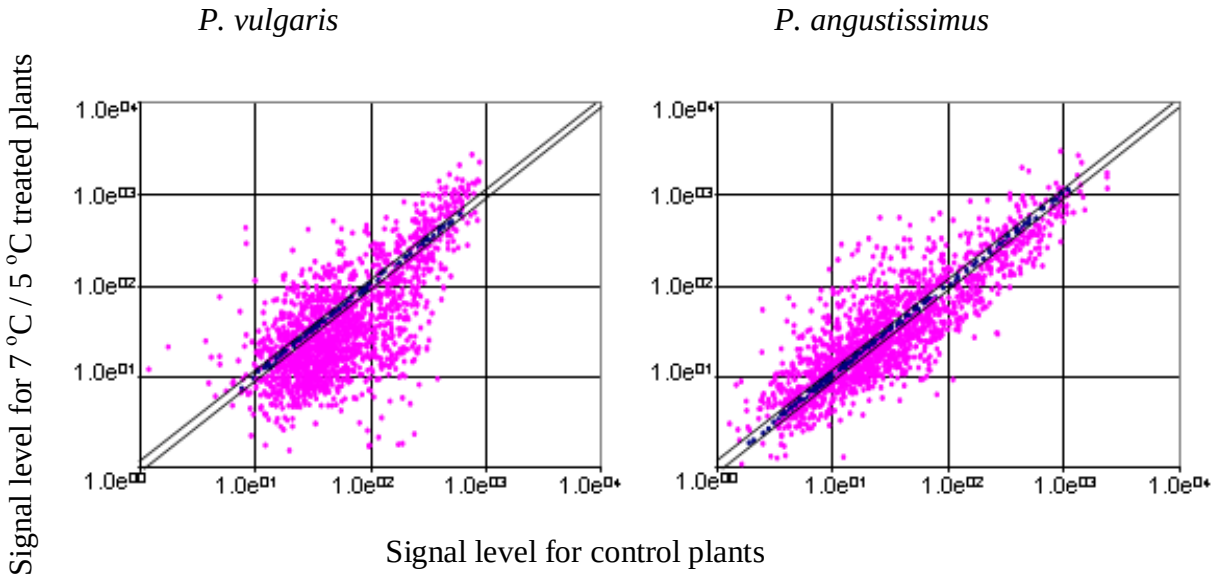

Figure 4.1. Scatterplots of normalized gene expression values in chilled *P. vulgaris* and *P. angustissimus.*

Normalized expression value for 1672 genes on a macroarray probed with cDNA from chilled (7 °C / 5 °C) plants (vertical axes) vs. the equivalent value for control (23 °C / 18 °C) plants (horizontal axes) for *P. vulgaris* (left) and *P. angustissimus* (right). Both axes are logarithmic. Diagonal lines indicate 95% confidence intervals.

gene expression response, as gene expression levels were distributed in a more linear fashion compared to *P. vulgaris*.

Analysis of clones represented on the array is presented as a ratio of normalized pooled specific spot hybridization intensity to normalized average total spot hybridization intensity across the two biological replicates in Appendix 1. Of the 453 genes that were found to be chilling responsive in *P. vulgaris* across two biological replicates, 77 were found to be up-regulated after three days of chilling, whereas 376 were found to be down-regulated. Comparatively, *P. angustissimus* had fewer (199) responsive genes after 72 hours of chilling. One hundred thirty-three of these *P. angustissimus* genes were found to be up-regulated, whereas 66 were found to be down-regulated.

A subset of 40 genes were selected from the *M. sativa* cDNA library on the basis of their putative involvement in stress response in plants. In chilled *P. vulgaris*, two genes of this subset were shown to be up-regulated and two were shown to be down-regulated, whereas in *P. angustissimus* all four of the chilling responsive genes of this subset were down-regulated (Table 4.4).

The available sequence information for some of the clones in the *M. sativa* cDNA library was not detailed enough to find suitable sequence alignment matches using BLAST. Of *P. vulgaris* genes with *M. sativa* homologues with poor sequence quality, two genes were up- and 43 genes were down-regulated over two biological replicates. Of *P. angustissimus* genes with *M. sativa* homologues with poor sequence quality, three genes were up- and one gene was down-regulated 3x over two biological replicates. Another limitation to further analysis came from the *M. sativa* gene

sequences aligning to genes with unknown function. Of *P. vulgaris* genes with unknown function, one gene was up- and 37 genes were down-regulated over two biological replicates. While for *P. angustissimus*, six genes with unknown function were up- and two were down-regulated over two biological replicates.

4.3.2 GO analysis of up-and down-regulated genes in chilled *P. vulgaris* and *P. angustissimus*

Best BLAST hits for *M. sativa* sequences of genes that were found to be transcriptionally up- or down- regulated in chilling in *P. vulgaris* and *P. angustissimus* were assigned Gene Ontology (GO) annotation for cellular localization, molecular function, and biological process using GO-Slim bioinformatic software (http://www.*Arabidopsis* .org/tools/bulk/go/index.jsp) (Tables 4.4, 4.5, and 4.6).

4.4 Discussion

4.4.1 Transcriptional responses to three days of low temperature treatment in *P. vulgaris* and *P. angustissimus*

Using this approach, the chilling sensitive cultivated species *P. vulgaris* was found to have a greater transcriptional response to three days of chilling compared to the slightly more chilling tolerant wild bean species *P. angustissimus*. The cultivated bean species, *P. vulgaris*, showed 83% of the chilling responsive genes being down-regulated after three days of chilling, which suggests that this species is either turning-down the expression of many genes or failing to sustain gene expression levels. The wild bean species, *P. angustissimus*, showed 33% of its chilling responsive genes to be down-regulated. This greater transcriptional response displayed by *P. vulgaris* is perhaps

Table 4.4. Expression ratios of genes specifically selected for analysis using macroarray.

Clone	Description	*P. vulgaris*	*P. angustissimus*
Ms02_01g06	apical meristem (NAM) family protein (RD26) [*Arabidopsis thaliana*]	0.39	1.11
Ms02_04g02	reaction associated Ca2+-binding protein [*Phaseolus vulgaris*]	1.68	1.27
Ms02_08h02	decarboxylase family protein [*Arabidopsis thaliana*]	0.86	0.52
Ms02_10a10	Photosystem I reaction center subunit IV A, chloroplast precursor (PSI-E A) [*Nicotiana tabacum*]	0.65	0.86
Ms02_10c11	GmMYB29A1 [*Glycine max*]	0.57	0.89
Ms02_10d07	copia-like polyprotein [*Lycopersicon esculentum*]	0.89	1.41
Ms02_12a12	dehydrin-like protein, CAS18 [*Medicago sativa*]	0.88	0.49
Ms02_13d11	ras-like small GTP binding protein [*Zea mays*]	0.72	1.17
Ms02_15g08	**leucine zipper protein [*Arabidopsis thaliana*]**	**1.77**	**0.28**
Ms03_06a10	kinase family protein [*Arabidopsis thaliana*]	0.91	0.86
Ms03_06g07	cold acclimation responsive protein BudCAR3 [*Medicago sativa*]	0.63	0.81
Ms03_07d08	acclimation protein WCOR413-like protein [*Oryza sativa*]	1.30	1.50
Ms03_11e08	probable bHLH transcription factor [imported] - *Arabidopsis thaliana*	0.52	1.73
Ms03_11e11	transcriptional activator CBF1-like protein [*Arabidopsis thaliana*]	0.84	0.87
Ms03_12e09	RING-H2 finger protein [*Oryza sativa*]	0.90	2.39
Ms03_14c04	responsive element binding factor 5 (ATERF5) [*Arabidopsis thaliana*]	0.66	1.77
Ms03_14d12	**glycine rich protein [*Daucus carota*]**	**3.95**	**0.21**
Ms03_15b04	Stress-related protein (PvSRP) [*Phaseolus vulgaris*]	0.83	0.41
Ms03_33e12	***P.vulgaris* mRNA for proline-rich protein (*PvPRP1*)**	**4.18**	**1.57**
Ms03_33f06	similar to AP2 domain containing protein RAP2.5[*Arabidopsis thaliana*]	0.59	1.29
Ms03_33g12	dnaK-type molecular chaperone precursor, mitochondrial [*Phaseolus vulgaris*]	1.11	0.89
Ms03_37g01	G protein beta subunit-like [*Medicago sativa*]	0.65	0.89
Ms03_38b12	phosphoinositide-specific phospholipase C [*Glycine max*]	0.69	1.26

Ms03_38c02	hypothetical protein [*Plasmodium yoelii yoelii*]	0.72	2.90
Ms03_39c02	probable lectin 2 precursor [*Medicago sativa*]	0.69	0.45
Ms03_44f12	dehydration responsive element binding protein GhDREB1A [*Gossypium hirsutum*]	1.24	0.87
Ms03_45c01	**AP2 domain transcription factor-like [*Arabidopsis thaliana*]**	**2.73**	**0.32**
Ms03_49a12	osmotic stress-induced zinc-finger protein [*Nicotiana tabacum*]	0.46	0.73
Ms03_49g10	probable zinc finger protein SCOF-1, cold-inducible [*Glycine max*]	0.68	0.40
Ms03_52a02	**low temperature and salt responsive protein LTI6B [*Arabidopsis thaliana*]**	**0.33**	**0.31**
Ms03_58a10	hydroxyproline-rich glycoprotein [*Sorghum bicolor*]	1.04	0.94
Ms03_60a12	transcription factor (CBF-B/NF-YA) family protein [*Arabidopsis thaliana*]	0.85	1.10
Ms03_60c02	dehydrin-like protein, CAS18 [*Medicago sativa*]	1.51	0.61
Ms03_64c06	protein kinase, putative / CDPK, putative [*Arabidopsis thaliana*]	0.81	1.84
Ms03_66c02	dehydrin-like protein [*Medicago sativa*]	1.76	1.46
Ms03_71e09	calcium/calmodulin-dependent protein kinase CaMK [*Oryza sativa*]	0.54	0.72
Ms03_75b07	Ca2+-binding protein (EF-Hand superfamily) [*Nicotiana tabacum*]	1.03	1.21
Ms03_75b11	cold-induced protein CAP160 [*Spinacia oleracea*]	0.80	1.44
Ms03_77h02	serine/threonine protein kinase [*Oryza sativa* (japonica cultivar-group)]	1.00	1.07
Ms03_78f01	**protein kinase MMK4, cold- and drought-induced [*Medicago sativa*]**	**0.20**	**2.23**

Each pooled clone percent of average clone hybridization intensity ratio is presented as an expression of average spot intensities of hybridization of transcripts from chilled plants over average spot intensities of hybridization of transcripts from non-chilled chilled plants. Genes in bold are discussed specifically in the text. Results averaged over two biological replicates.

Table 4.5. GO analysis of cellular component of genes found to be up- or down-regulated in three day chilled *P. vulgaris* and *P. angustissimus* interspecific macroarrays using *M. sativa* cDNAs.

GO Cellular Component	% up-regulated		% down-regulated	
	P. vulgaris	*P. angustissimus*	*P. vulgaris*	*P. angustissimus*
cell wall	5.9	0.0	1.7	2.2
chloroplast	9.8	5.1	7.9	15.2
cytosol	11.8	12.2	5.0	4.7
ER	0.0	1.0	2.5	0.0
extracellular	2.0	1.0	0.4	0.0
Golgi apparatus	2.0	2.0	3.3	2.2
mitochondria	15.7	3.1	5.0	4.3
nucleus	13.7	15.3	14.1	10.9
other cellular components	0.0	1.0	0.8	4.3
other cytoplasmic components	25.5	22.4	12.9	10.9
other intracellular components	25.5	22.4	18.7	8.7
other membranes	25.5	35.7	21.6	21.7
plasma membrane	2.0	2.0	2.9	2.2
plastid	5.9	3.1	2.5	4.3
ribosome	13.7	13.3	5.8	6.5
unknown cellular components	19.6	23.5	31.1	30.4

Table 4.6. GO analysis of molecular function of genes found to be up- or down-regulated in three day chilled *P. vulgaris* and *P. angustissimus* interspecific macroarrays using *M. sativa* cDNAs.

GO Molecular Function	% up-regulated		% down-regulated	
	P. vulgaris	*P. angustissimus*	*P. vulgaris*	*P. angustissimus*
DNA or RNA binding	5.5	8.3	10.2	13.0
hydrolase activity	9.1	11.1	10.9	8.7
kinase activity	7.3	1.9	6.9	4.3
nucleic acid binding	5.5	2.8	4.4	2.2
nucleotide binding	7.3	6.5	5.5	6.5
other binding	7.3	11.1	12.0	10.9
other enzyme activity	20.0	8.3	13.1	15.2
other molecular functions	20.0	2.8	2.6	2.2
protein binding	18.2	15.7	8.0	6.5
receptor binding or activity	1.8	1.9	0.7	0.0
structural molecule activity	12.7	11.1	6.2	10.9
transcription factor activity	5.5	11.1	7.7	4.3
transferase activity	14.5	10.2	12.0	10.9
transporter activity	3.6	5.6	6.6	4.3
unknown molecular functions	1.8	17.6	20.8	26.1

Table 4.7. GO analysis of biological process of genes found to be up- or down-regulated in three day chilled *P. vulgaris* and *P. angustissimus* interspecific macroarrays using *M. sativa* cDNAs.

GO Biological Process	% up-regulated		% down-regulated	
	P. vulgaris	*P. angustissimus*	*P. vulgaris*	*P. angustissimus*
cell organization and biogenesis	9.6	5.9	11.6	10.4
developmental processes	11.5	6.9	5.4	8.3
DNA or RNA metabolism	1.9	1.0	3.5	2.1
electron transport or energy pathways	3.8	2.0	3.1	8.3
other biological processes	15.4	7.9	9.3	4.2
other cellular processes	48.1	53.5	47.7	37.5
other metabolic processes	50.0	57.4	48.5	33.3
protein metabolism	25.0	22.8	19.8	18.8
response to abiotic or biotic stimulus	28.8	11.9	7.8	18.8
response to stress	19.2	6.0	7.4	14.6
signal transduction	5.8	4.0	3.9	4.2
transcription	9.6	13.9	8.9	6.3
transport	5.8	6.9	7.0	8.3
unknown biological processes	19.2	21.8	26.4	31.3

indicative of the perturbed state of this plant species after three days of chilling compared to *P. angustissimus*.

The inability of *P. vulgaris* to sustain transcriptional regulation on the third day of chilling may be indicative of the inability to maintain normal gene expression levels as opposed to a concerted effort to transcriptionally repress gene expression. Further support of this hypothesis is that *P. vulgaris* showed lower transcript levels of genes that have been shown to be beneficial to the survival of other species under chilling conditions (Abba et al., 2006). The *P. vulgaris* plants down-regulated genes that are found to be related to dehydration stress in other species, including dehydrin-coding genes. Amphipathic alpha helices predicted to be formed by dehydrins and are believed to interact with membrane lipids in a similar manner to amphipathic helix structures of apolipoproteins, which potentially serve a protective role during dehydration, as well as osmotic and low temperature stress (Close, 1997; Wisniewski et al., 1999).

Dehydrins belong to the LEA (late embryogenic abundant) II protein family, a family that includes *COR* (cold regulated) genes. *LEA*/*COR* genes are induced by drought, high salinity, and ABA (Thomashow, 1999) and are believed to play a role in ameliorating the damaging effects of cellular dehydration. *LEA*- and *COR*-like genes are typically expressed when plants are subjected to low temperature and dehydration stresses and are found to positively correlate with increased ability to survive low temperature stress (Thomashow, 1999; Kume et al., 2005). The down-regulation of genes that code for proteins of such critical function suggests that *P. vulgaris* is not responding to low temperatures in a way that could maintain normal (non-chilled) levels of expression of genes that would enable it to cope with the damaging effects of low

temperatures. Results of physiological experiments above (see 3.3.2) showed that there was a loss in water content over the first 72 hours of low temperature exposure, and thus the inability of 72 hour chilled *P. vulgaris* to maintain gene expression levels of dehydrins may indicate that this species is not coping with this loss in water content.

Genes that code for cytochrome P450s were also down-regulated in each biological replicate in *P. vulgaris*. Cytochrome P450s are members of a superfamily of monooxygenases, which are enzymes that catalyze reactions in cells, including enzymatic reactions involving lipid metabolism (Narusaka et al., 2004). Since cell membrane phospholipid integrity is crucial to normal cellular function under low temperature conditions, expression of cytochrome P450 genes may be necessary to maintain membrane lipid profiles. In not maintaining normal expression levels of cytochrome P450, *P. vulgaris* could be suffering a loss in membrane integrity, which could be linked to electrolyte leakage and, as seen in section 3.3.2, loss of water content. Genes that code for cytochrome P450s were seen to be up-regulated in low temperature treated *P. angustissimus*, which may contribute to the ability of these plants to maintain water content levels in low temperature conditions as seen in section 3.3.2. While this particular cytochrome P450 has yet to be functionally explored in plant stress, other members of the superfamily have been linked to abiotic stress via their transcriptional up-regulation in response to reactive oxygen species (ROS) in *Arabidopsis* (Narusaka et al., 2004). Down-regulation of these genes in *P. vulgaris* is further indicative of the inability of that species to adjust properly to chilling temperatures, while the ability of *P. angustissimus* to not be damaged at chilling temperatures may be related to the maintenance of the expression levels of this gene.

4.4.2 Transcriptional changes of 40 pre-selected genes in *Phaseolus*.

The vast majority of the 40 genes known to be linked to surviving low temperatures and purposely selected for the array experiments, were not found to be consistently responsive to low temperatures across two biological replicates, as only eight genes (four in *P. vulgaris* and four in *P. angustissimus*) of this group showed consistent regulation across two biological replicates (Table 4.4). The genes that showed consistent up- or down-regulation in chilled *P. vulgaris* were Ms03_14d12, Ms03_33e12, Ms03_52a02, and Ms03_78f01, and the genes that showed consistent up- or down-regulation in chilled *P. angustissimus* were Ms02_15g08, Ms03_14d12, Ms03_45c01, and Ms03_52a02. The remainder of this 40 gene subset included transcription factors, such as the cold-inducible zinc-finger DNA binding protein SCOF (Xiong et al., 2002) which was found to be strongly up-regulated after 12 and 24 hours of 4^{o}C in soybean (Kim et al., 2001), as well as members of the well established CBF (C-repeat binding factor) low temperature response pathway (Thomashow, 2001). CBF regulation has been shown to have different response to cold depending on the temperature tolerance of the plant species. For example, *Arabidopsis*, which can better survive low temperatures, was found to rapidly up-regulate CBF upon low temperature exposure and sustained expression levels over 24 hours of chilling, while tomato, a low temperature sensitive species, did not strongly up-regulate CBF under the same conditions (Jaglo et al., 2001). Genes coding for members of the calcium signaling pathway, which is known for its involvement in low temperature response (Monroy and

Dhindsa, 1995) and which were among the 40 gene subset, were also not found to be consistently transcriptionally regulated across two biological replicates. Calcium signatures have been found to confer low temperature tolerance in a variety of plant species (Knight and Knight, 2001).

Genes related to the synthesis of the osmolyte proline (Xiong et al., 2002), were also specifically selected from the *M. sativa* library for examination regarding responsiveness to low temperatures in these *Phaseolus* species. In *P. vulgaris*, one proline-related gene, *PvPRP1* (*M. sativa* clone ID Ms03_33e12), was found to be consistently transcriptionally up-regulated across two biological replicates, while similarly treated *P. angustissimus PvPRP1* was not consistently regulated (Table 4.4). Increases in proline levels have been shown to protect against osmotic water loss as a result of low temperatures in Puma rye (*Secale cereale* L.; Koster and Lynch, 1992), while proline levels have not been shown to be strongly induced by low temperatures in chilling sensitive pea (*Pisum sativum* L.) or mung bean (*Vigna radiata* L.) (Rosinger et al., 1984). Additionally, proline-rich proteins have been shown to be transcriptionally responsive to drought and salt stresses (He et al., 2002) which have similar impacts to low temperature stress. While some proline-related genes were not up-regulated under chilling conditions in these *Phaseolus* species, the up-regulation of this particular *PvPRP1* in *P. vulgaris* may warrant further inquiry into the role this gene plays in low temperature response.

In addition to *PvPRP1*, a gene coding for a glycine-rich protein (Ms03_14d12) also demonstrated up-regulation in *P. vulgaris* under chilling conditions, while this same gene did not show consistent regulation in *P. angustissimus* (Table 4.4). Up-

regulation of glycine-rich proteins have been found to have beneficial roles in some plant species under cold acclimation conditions, as glycine-rich proteins play roles in RNA chaperoning in low temperature conditions (Kim and Kang, 2006).

In *P. vulgaris*, a gene that codes for a cold- and drought-responsive protein, mitogen activated protein kinase MMK4 (Ms03_78f01) was found to be down-regulated on the array (Table 4.4). The down-regulation of this gene is in contrast with studies of the expression of this gene in *M. sativa*, as Jonak et al. (1996) showed that this gene was up-regulated within 20 min of low temperature exposure in alfalfa in an abscisic acid-independent manner, and had sustained high transcript levels over 24 hours of cold temperature exposure. This suggests that in *P. vulgaris* this gene was not being expressed in a manner similar to other low temperature tolerant species, and may be an indicator of stress in this bean species. In *P. angustissimus*, this gene did not show consistent up- or down-regulation in chilled *P. angustissimus*, which may indicate that that this bean species was not responding similar to *P. vulgaris*.

In *P. angustissimus*, homologues to genes that code for an AP2 transcription factor (Ms03_45c01), a low temperature and salt responsive protein (Ms03_52a02), a leucine zipper protein (Ms02_15g08), and a glycine-rich protein (Ms03_14d12) were down-regulated (Table 4.4). Up-regulation of transcription factors containing AP2-type DNA binding motifs have been implicated in ameliorating the damaging effects of low temperature stress in plant species that respond to cold acclimation treatment (Shinozaki and Yamaguchi-Shinozaki, 2000). While a gene coding for an AP2-type DNA binding protein was found to be down-regulated in chilled *P. angustissimus* in these experiments, the same gene was not found to be up- or down-regulated in chilled *P.*

vulgaris, which may indicate neither of these bean species were responding to low temperatures in a stress responsive manner with regards to this gene. The low temperature and salt responsive protein coding transcript down-regulated in *P. angustissimus* is similar to the RCI (Rare Cold Inducible) type of membrane spanning proteins. RCI proteins are believed to provide membrane stability during dehydration stress (Capel et al., 1997) and a relative of RCI in the chilling sensitive rice (OsLti6a; *Oryza sativa*) has been shown to be up-regulated in low temperatures (Morsy et al., 2005). While a gene coding for an RCI protein was found to be down-regulated in chilled *P. angustissimus* in these experiments, the same gene was not found to be up- or down-regulated in chilled *P. vulgaris*, which may indicate neither of these bean species were responding to low temperatures in a stress responsive manner with regards to this gene. Leucine zipper proteins are involved in signal transduction pathways in an ABA-dependant manner in *Arabidopsis* plants under drought and salt stress conditions (Uno et al., 1997) and were found to be strongly up-regulated in 2- through 5-day drought stressed *P. vulgaris* root tissues (Rodriguez-Uribe and O'Connell, 2006). While a gene coding for a leucine zipper protein was found to be down-regulated in chilled *P. angustissimus* in these experiments, the same gene was not found to be up- or down-regulated in chilled *P. vulgaris*, which may indicate that the aerial tissues of these bean species are not expressing leucine zipper coding genes similar to the root tissues of *P. vulgaris*. Glycine-rich proteins are believed to act as RNA chaperones during low temperature response in *Arabidopsis* (Karlson et al., 2002), and are believed to enhance freezing tolerance (Kim and Kang, 2006). While a gene coding for a glycine-rich protein was found to be down-regulated in chilled *P. angustissimus* in these

experiments, the same gene was found to be up-regulated in chilled *P. vulgaris*, which may indicate that *P. vulgaris* was responding to low temperatures in a stress responsive manner.

4.4.3 Gene ontology of transcriptionally responsive genes in *Phaseolus* and comparison to other *Phaseolus* genomic studies.

The Gene Ontology (GO) consortium has developed a searchable database that uses controlled vocabularies to assign functional roles for three structured ontologies. Using these vocabularies, an understanding of cellular components, molecular functions, and biological processes of gene products can be discussed. GO analyses are helpful in providing a functional context for the results for macroarrays in a species-independent manner. For instance, comparing GO cellular components of gene products of down-regulated *P. vulgaris* genes with down-regulated *P. angustissimus* genes (Table 4.5), it is shown that genes involved in the chloroplast represent more in *P. angustissimus* than in *P. vulgaris*. In Table 4.6, the proportion of up-regulated genes involved in the GO molecular functions for transcription factor activity is greater in chilled *P. angustissimus* than *P. vulgaris,* which could be indicative of the activation of gene signaling cascades in the wild bean species. In Table 4.7, the GO biological process analysis shows a greater proportion of genes related to related to stress were up-related in *P. vulgaris* compared to *P. angustissimus*, which further supports the hypothesis that *P. angustissimus* survives better in low temperature conditions than *P. vulgaris*. From this type of analysis, it is difficult to infer whether down-regulation is beneficial or a symptom of damage, but identifying gene populations that are related to

gene ontologies reveals insight into the changes seen in bean during low temperature exposure.

Using the results of the GO analysis, comparisons can be made between the results of the interspecific macroarray experiments presented here and analyses of cDNA libraries developed using similarly non-chilled and three-day chilled *P. vulgaris* and *P. angustissimus* (Vijayan et al., 2008). In general, the results of the interspecific macroarray experiments are similar to those of the subtractive suppression hybridization (SSH) study, in that the proportions of genes in the various GO categories were similar between *P. vulgaris* and *P. angustissimus*. One of the key findings in the SSH study was that genes belonging to the 'structural molecule activity' GO category were down-regulated in *P. angustissimus* to a larger degree than in *P. vulgaris*, and these trends were also seen in the interspecific macroarray experiments presented here. In cases where *P. vulgaris* and *P. angustissimus* show dissimilarity in GO category proportions in the interspecific macroarray experiments, the results appear to show that *P. vulgaris* is enduring, and transcriptionally responding to, a stress event, whereas similarly treated *P. angustissimus* is not behaving in a similar manner. Specifically, genes belonging to the 'response to stress' GO category showed as greater % of down-regulation in the relatively more chilling tolerant *P. angustissimus* in the interspecific macroarray experiments as seen in the SSH experiments. Furthermore, using the interspecific macroarray experiments, genes belonging to the 'response to stress' and the 'response to biotic and abiotic stimulus' GO category were found to be up-regulated in a greater proportion in the chilling susceptible *P. vulgaris* than the relatively chilling tolerant *P. angustissimus*, particularly in regard to the former category, compared to SSH

experiments. This result of the macroarray experiments suggests that *P. vulgaris* is transcriptionally responding to a perceived stress whereas *P. angustissimus* is not behaving in a similar manner. Taken together, while differences are evident with regard to the proportion of genes being up- and/or down-regulated in certain GO categories, the results seen in Vijayan et al. (2008) show general congruity in that the proportions of *P. vulgaris* and *P. angustissimus* of most GO categories fall within the same range.

Differences between the results seen here and the results of Vijayan et al. (2008) could partially be considered in the context that one approach was the use of interspecific hybridization using a small subsample of a cDNA library of cold acclimated *M. sativa*, thereby predetermining the range and types of genes available for hybridization to *Phaseolus* cDNAs, whereas SSH cDNA libraries of *Phaseolus* do not share these same restrictions. Thus, particularly when assessing the results of *Phaseolus* GO annotations in the experiment presented here, one must be cognizant that the distribution of the *M. sativa* GO annotations being sampled on the array (see Table 4.1) limited the kinds of genes that were being assayed. In this context, some results are even more striking, such as in the disparity between the proportion of genes involved in the GO biological processes of 'response to abiotic and biotic stimulus' and 'response to stress'. In *P. vulgaris*, despite 'response to abiotic and biotic stimulus' and 'response to stress' representing only 6.3% and 5.8% of the annotations sampled, respectively, genes of these categories were found to be up-regulated 28.8% and 19.2%, respectively, whereas in *P. angustissimus*, genes of these annotations were up-regulated 11.9 and 6.0%, respectively.

4.4.4 Factors to consider when interpreting the results of these array experiments

Among the factors to be considered when assessing the results of the array experiments is that the sequence data for all clones featured on the array were not of uniformly high quality. Many potentially key hybridization events were not examined further on the basis of the low fidelity of the sequence data. Many of the *M. sativa* cDNAs used to spot the macroarray did not correspond to unique genes, and some of the cDNAs spotted on the array could have been paralogues, thus the number of actual genes assayed is lower than the number of cDNAs spotted. In the case of the CAS18 dehydrin, which was spotted on the array 12 times, *P. vulgaris* hybridizations showed consistent trends of down-regulation in 10 of 12 of those spots in the first biological replicate, and similar trends in down-regulation in 8 of 12 spots in the second biological replicate. Such spot replicates generally support the robustness of the macroarray data, though a more sensitive hybridization assay method may be necessary to obtain results that are closer to uniformity across spot replication.

There are many potential reasons for the inconsistencies seen between biological replicates in these macroarray experiments. Biological variation is one potential reason for the differences seen between biological replicates, but it should also be noted that the conditions under which the two experiments were performed were perhaps slightly different, as cDNA hybridization conditions were optimized for, and the first cDNA hybridization was conducted in, Ste-Foy, Quebec, whereas cDNA hybridizations for the second biological replicate were conducted in Saskatoon, Saskatchewan. Subtle differences in cDNA hybridization conditions may introduce variability, and results of

these cDNA hybridization experiments need to be interpreted with knowledge of the context in which the experiments were conducted.

It should also be noted that adjusting the cutoff values demarcating up- and down-regulated genes (which were set to three-fold) had little impact of the proportion of genes found up- and down-regulated in response to chilling in these bean species in either biological replicate. However, there are some cases where the three-fold cutoff may exaggerate changes in gene expression. For example, a gene coding for a heat shock protein was found to be up-regulated 27.4 times in the first biological replicate and up-regulated 2.9 (below the three-fold cutoff) in the second biological replicate. While the pooled average up-regulation is well above three-fold, the examination of the two biological replicates separately reveals variation in degree of fold change. Results of these experiments should be evaluated with the knowledge that the possibility exists for one particular replicate having a strong influence on pooled fold change values.

Additionally, the spotted cDNAs were derived from cold acclimated *M. sativa* crowns, and thus many genes that may be uniquely related to *Phaseolus*-specific low temperature response could be missing from this array. One can expect that the types of transcripts that were harvested from *M. sativa* plants exposed to two weeks of 2 ^{o}C / -2 ^{o}C may be quite different from those harvested from three day chilled *Phaseolus* at 7 ^{o}C day / 5 ^{o}C. A prudent future direction would be to use a genomic *Phaseolus* library or sequenced cDNAs harvested from comparably chilled *Phaseolus* to assay within this genus.

Using a *M. sativa* cDNA library to print membranes for hybridizing cDNAs of non-chilled and chilled *P. vulgaris* and *P. angustissimus* plants, a number of genes with

known function were identified as chilling-responsive in each species over two independent biological replicates. Although all *M. sativa* cDNAs for these macroarray experiments were selected from a cDNA library of cold acclimated *M. sativa*, many known chilling responsive genes were not identified as consistently responsive in these *Phaseolus*. One explanation for the disparity is that the cold acclimation conditions under which the *M. sativa* cDNA libraries were developed were considerably different than the 72 hour low temperature exposed *Phaseolus* plants used in the experiments, as alfalfa plants were treated to two weeks of 2 ^{o}C day / -2 ^{o}C night. Additionally, alfalfa is a winter hardy perennial plant capable of surviving temperatures well below -10 ^{o}C, whereas both *Phaseolus* species do not survive one night of -7 ^{o}C in the field (Balasubramanian, 2002), so differences in gene regulation patterns could be anticipated. Furthermore, only a subset of the *M. sativa* cDNA library was sampled for the array, which limits the number and types of genes assayed in these bean species.

4.4.5 General conclusions for interspecific macroarray experiments

Using a macroarray to compare and contrast gene expression in chilled *P. vulgaris* and *P. angustissimus*, it was shown that these two species responded to low temperatures differently. Macroarray experiments showed that *P. vulgaris* demonstrated greater overall transcriptional response on the third day of chilling compared to *P. angustissimus*, and that the transcriptional response was largely a shift towards down-regulation. Comparatively, *P. angustissimus* demonstrated up-regulation for the majority of the genes that responded to three days of chilling. One similarity between these two species in these experiments is that many of the transcripts that were found to be transcriptionally responsive in each species after pooling normalized

hybridization intensities, were not consistently transcriptionally responsive in each of two separate biological replications.

While there are many factors one needs to consider when conducting interspecific cDNA hybridizations, one can conclude that *P. vulgaris* and *P. angustissimus* behave differently after three days of chilling temperatures with regards to the expression of the genes sampled, as results showed that chilling sensitive *P. vulgaris* plants showed that 453 of these genes were chilling responsive (77 up-regulated and 376 down-regulated), whereas the chilling tolerant *P. angustissimus* plants showed only 199 of these genes were chilling responsive (134 up-regulated and 65 down-regulated). Analysis of the molecular characteristics of chilling responsive genes showed that *P. vulgaris* plants experiencing stress after three days of chilling temperature exposure more than *P. angustissimus*. However, given the caveats involved with conducting interspecific macroarray cDNA hybridizations, there is a necessity to confirm the expression of a sample of genes using more sensitive measures of gene expression, such as RT-PCR. Results of RT-PCR confirmation experiments reveal insights into the reliability of interspecific cDNA macroarray hybridizations.

5. Characterization of expression patterns of *Phaseolus* genes

5.1 Introduction

Despite the ability of plants to cope with a multitude of environmental stresses, including cold, drought, and salinity, crop losses worldwide are a result of the inability of cultivated plant species to adjust to these stresses (Boyer, 1982). Many major crop yields are often reduced by 50% or more as a result of environmental stresses (Bray et al., 2000). Comparatively, yield loss as a result of pathogens normally ranges from 10-20% on average (Kreps et al., 2002).

Severe damage can result from exposing sensitive plant species to low temperatures for a prolonged period of time. In many species, changes in gene expression and subsequent protein synthesis have resulted in plants with the ability to cope with the detrimental impacts of low temperature stresses (Wallis et al., 1997). Many low temperature regulated genes have been identified and characterized in a variety of plant species, and many investigations have focused on gene expression analysis of stress-response mechanisms (Collinge and Boller, 2001; Dean et al., 2002 Cattivelli and Bartels, 1992; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2007; Kim et al., 2007). Gene expression analyses of low temperature treated plants have facilitated a more comprehensive understanding of stress responses in a variety of plant species. Multiple gene signaling networks have been isolated and described. Through these studies, numerous novel stress-responsive genes have been discovered,

revealing significant cross-talk between stress responses (Shinozaki and Yamaguchi-Shinozaki, 2000).

In Chapter 4, interspecific macroarray experiments identified numerous genes that were shown to be chilling responsive in three day chilled *Phaseolus vulgaris* and *Phaseolus angustissimus*. It is important to confirm the expression of a sample of genes identified on arrays to assess the reliability of the results of array experiments using more accurate measures of gene expression (eg. RT-PCR). Among the genes that were identified as chilling responsive and selected for confirmation using RT-PCR in these bean species, many are known to play roles in stress responses in plants, including those that code for cytochrome P450s (*CYP72A14*), heat shock proteins (HSP), gamma-aminobutyric acid A receptors (GABAr), abscisic acid activated proteins (ABAp), basic helix-loop-helix (bHLH) proteins, pyruvate decarboxylase isozymes (PyD), and constitutive photomorphogenic interactive proteins (CIP). Cytochrome P450 genes code for enzymes involved in lipid, terpinoid, phenolpropanoid, phenolic, and alkaloid metabolism (Narusaka et al., 2004), whereas HSPs are molecular chaperones that stabilize protein structure (Schöffl et al., 1998). GABA is known to accumulate in plants in response to stress, and is thought mitigate water stress by functioning as an osmolyte and, possibly, as a signaling molecule because of the presence of GABA receptors in many plant species (for review, see Kinnersley and Turano, 2000). Abscisic acid is an important plant hormone involved in stress response that up-regulates many proteins involved in coping with many environmental stresses (Zhu, et al., 2002). Transcription factors of the bHLH family have been shown to be critical for the up-regulation of many genes involved in low temperature response in plants

(Chinnusamy et al., 2003) while PyDs have been shown to be involved in responses to anoxic conditions (Kursteiner et al., 2003). NFU2s and CIPs are involved in chloroplast biogenesis, as NFU2s are required for assembly of the Fe-S complex of photosystem I (Touraine et al., 2004) and CIPs are positive regulators of chloroplast accumulation by interaction with constitutive photomorphogenic proteins (Eckardt, 2001**).**

In this work, expression patterns of genes were estimated using semi-quantitative RT-PCR over a time-course of low temperature exposure from 0 to 72 hours in order to confirm the expression patterns observed in interspecific macroarray cDNA hybridizations (see Chapter 4). These genes are homologous to *M. sativa* cDNA sequences that were identified using interspecific macroarray experiments. Furthermore, to better understand the low temperature expression of the genes selected, expression levels of each gene was monitored over a time-course of chilling temperature exposure.. The expression levels of each gene at each time-point in this experiment were measured in relation to the expression of the reference gene beta-tubulin. The results presented highlight variations in gene expression over a time-course of chilling temperature exposure and across biological replicates that, in many cases, do not reflect the results of macroarray experiments.

5.2 Materials and methods

5.2.1 Plant materials and growth conditions

Cultivated common bean (*Phaseolus vulgaris* L. cv ICA Pijao) and wild narrow leaf bean (*P. angustissimus* L. PI535272) were germinated and grown for 21 days as described in 3.2.1. Plants selected for chilling were transferred to a growth chamber, preset to 7 ^{o}C day / 5 ^{o}C night with the same light conditions, for 72 hours. Plants to be

used in RT-PCR reactions were grown as described in 3.2.1, and aerial tissues of plants chilled for 0, 0.25, 0.50, 1, 2, 12, 24, and 72 hours were collected for each species. Plant tissues from each time-point were immediately frozen in liquid nitrogen and stored at -80 ^{o}C until RNA preparation for RT-PCR experiments.

5.2.2 RT-PCR analysis

Total RNA was extracted and purified as described in 4.2.2 from all tissue samples. cDNAs were generated from 1.0 µg of total RNA with 400 nM of oligo dt primer. Reverse transcription was done using M-MLV reverse transcriptase (Invitrogen, Carlsbad, California, USA) according to manufacturer's instructions. A 1.0 µL aliquot of the total RT reaction volume (50 µL) was used as template in a 25 µL semi-quantitative RT-mediated PCR amplification reaction. Primer sequences used in RT-PCR experiments are listed in Table 5.1 and an overview of genes being studied, including observed fold change in macroarray experiments and fragment size, are shown in Table 5.2. PCR primers were derived from sequence data obtained from cDNA libraries of *P. vulgaris* and *P. angustissimus* (Vijayan et al., 2008) using the PrimerQuest software provided by Integrated DNA Technologies (Coralville, Iowa, USA). Genes to confirm macroarray experiments and to explore expression in greater detail were selected on the basis of nucleic acid sequence homology between the *M. sativa* cDNA spot sequence and *Phaseolus* cDNA sequences using a cutoff of 80% identity match where applicable. Each 25 µL PCR reaction was carried out using 0.2 µM of species-specific gene-of-interest primers (forward and reverse) and 0.2 µM species-specific beta-tubulin primers (forward and reverse) in 1X Taq Buffer

Table 5.1. Primer sequences used for RT-PCR experiments to confirm results from the macroarray experiment in Chapter 4.

Species	Reference Gene	Forward Primer Sequence 5'-3'	Reverse Primer Sequence 5'-3'
P. vulgaris	beta-tubulin	ttctcgtgggtctcagcagtatgt	actgctactcaccctcctaaaca
P. angustissimus	beta-tubulin	ttctcgtgggtctcagcagtatgt	actgctactcaccctcctaaaca

Species	*Medicago sativa* Homologue	Forward Primer Sequence 5'-3'	Reverse Primer Sequence 5'-3'
P. vulgaris	HSP 83	tgtgacaagggaraccgatggtga	tcaagtccttcaatcgacgctcct
P. vulgaris	GABAr	ttgcaagaatgctgtctcgccatc	tccaaccaccactcaatacctgct
P. vulgaris	ABA protein	agccaacagagcaaaggatctcca	acttccaaactccggtttgctgtc
P. vulgaris	Cytochrome P450	gcaccatacgtggttgaagagtg	ctccttattggaagcaaagatggc
P. angustissimus	Cytochrome P450	ggtgattatttgtccgtgaggtcc	tgctccagtcactgtgttcactct
P. angustissimus	Py D	agctgaaattgcccaagggatgtg	aaggcaatcactcgcttctcagga
P. angustissimus	bHLH	taactcgcagccatgagtgagca	ttcaagagctgctttggccgtttc
P. angustissimus	NFU2	cgacgctcaatgcccagtttcatt	cattgcagatggtggaaatgtggc
P. angustissimus	CIP1	cttcctcaaagccacttcctctttc	gggcgatattgaggttgtaaaggg

Table 5.2 List of genes studied using RT-PCR experiments. Observed fold changes in macroarray experiments are shown where applicable.

Species	Gene homologue	Observed fold change on macroarray	Expected RT-PCR fragment size
P. vulgaris	HSP 83	up-regulated 15.15x	104
P. vulgaris	GABAr	down-regulated 6.25x	110
P. vulgaris	ABA protein	up-regulated 3.49x	106
P. vulgaris	Cytochrome P450	down-regulated 18.1x	120
P. vulgaris	beta-tubulin	n/a	332
P. angustissimus	Cytochrome P450	up-regulated 4.29x	120
P. angustissimus	Py D	down-regulated 3.78x	121
P. angustissimus	bHLH	up-regulated 5.48x	104
P. angustissimus	NFU2	down-regulated 1.3x	100
P. angustissimus	CIP1	down-regulated 6.0x	107
P. angustissimus	beta-tubulin	n/a	332

(GeneScript Corporation, Piscataway, New Jersey, USA) with 0.4 mM dNTPs. Sequences of beta-tubulin genes from *P. vulgaris* and *P. angustissimus* were used to design primers to follow expression of this reference transcript in species specific RT-PCR reactions. PCR reactions were separated on a 1% agarose gel containing 0.1 µg/µL ethidium bromide and visualized under UV light. The signal intensity of the amplified product was then estimated semi-quantitatively using the Alpha Imaging System (Alpha Innotech Corporation, San Leandro, USA) according to the manufacturer's instructions, and gene specific band intensity was calculated as a percent of the respective time-point beta-tubulin band intensity. Thirty-two cycles of PCR (94 ^{o}C for 0.5 min, 54 ^{o}C 0.4 min, 72 ^{o}C for 0.5 min) followed by 72 ^{o}C for 2.0 min was carried out for each PCR reaction. This cycle number was determined using test-PCR experiments of varying cycle numbers (including 28, 30, 32, 34, and 36 cycles) to ensure that gene amplification levels were below saturation levels while reference gene amplification levels were observable. Effective removal of genomic DNA from RT-PCR samples was confirmed by observing no bands in PCRs that were carried out as above using 1 µg of RNA (after DNAse I treatment and E. Z. N. A. clean-up) as template. Statistical analyses of gene expression levels were performed using SAS System 8.2 (SAS Institute Inc., Cary, North Carolina, USA), in which a GLM procedure was used to test for significant differences between quantified cDNA quantities. The contrast function was used to compare gene expression levels for pairs of specific time-points. Calculated P values of less than 0.05 were considered to be statistically significant.

5.3 Results

To evaluate expression levels of four *P. vulgaris* and five *P. angustissimus* genes over various durations of low temperature exposure, cDNA quantities were estimated as a percent of a species specific beta-tubulin reference gene. Expression levels of the mean of three biological replicates of selected *P. vulgaris* genes across various low temperature incubation durations are shown in Figure 5.1, and expression levels of three biological replicates of selected *P. angustissimus* genes across various low temperature incubation durations are shown in Figure 5.2.

5.3.1 Confirmation and characterization of gene expression patterns

In the interspecific macroarray experiment (see section 4.3), HSP83 had shown a high level of up-regulation following three days of chilling in hybridizations of cDNA from *P. vulgaris* plants for 72 hour chilled compared to non-chilled control plants using the interspecific macroarray. In contrast, gamma-aminobutyric acid receptor subunit epsilon (GABAr) appeared to be substantially down-regulated in the same interspecific macroarray experiment. Genes putatively coding for heat shock protein 83 (HSP83) and GABAr were shown to be up-regulated in samples of 24 hour chilled *P. vulgaris* plants compared to non-chilled control levels of expression, while maintaining expression levels not significantly different to non-chilled control levels in any of the other chilling time-points sampled across three biological replicates (Figure 5.1). In two of the three biological replicates of the RT-PCR experiments, HSP83 showed lower levels of expression at the 72 hours of chilling time-point compared to the non-chilled control. GABAr also showed lower levels of expression in the 72 hours of chilling

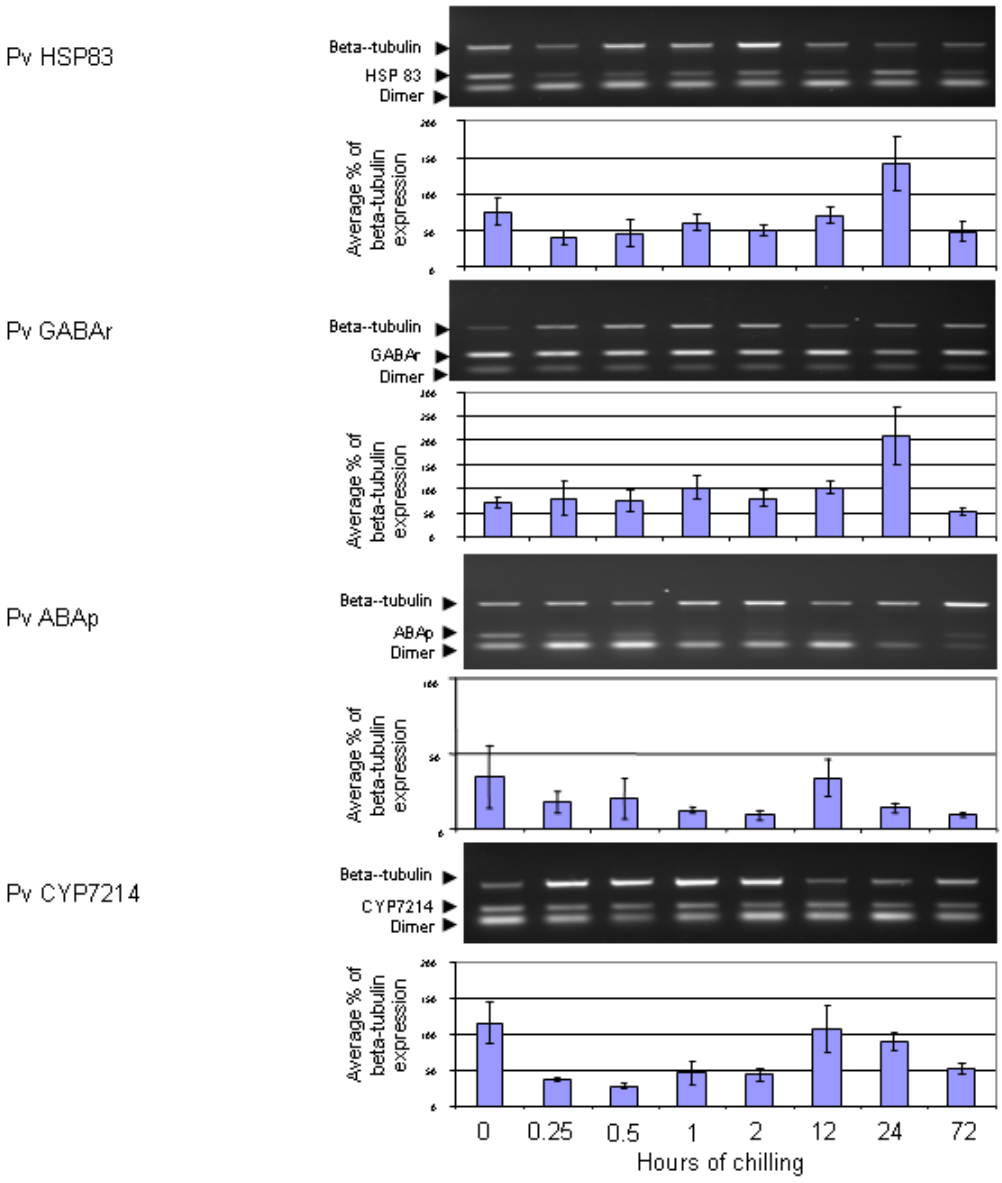

Figure 5.1. RT-PCR analysis of expression patterns of four *P. vulgaris* genes.
RT-PCR analysis was carried out for each gene using RNA extracted from tissue of plants harvested at 0, 0.25, 0.5, 1, 2, 12, 24, and 72 hours of exposure to 7 ^{o}C day / 5 ^{o}C night. Changes in gene expression were estimated relative to the expression of beta-tubulin, which was amplified in the same reaction (one example shown). Means and standard error for three biological replicates presented. HSP83, heat shock protein; ABA, abscisic acid associated protein; GABAr, gamma-aminobutyric acid A receptor; CYP7214, cytochrome P450. Gel images are of one biological replicate shown.

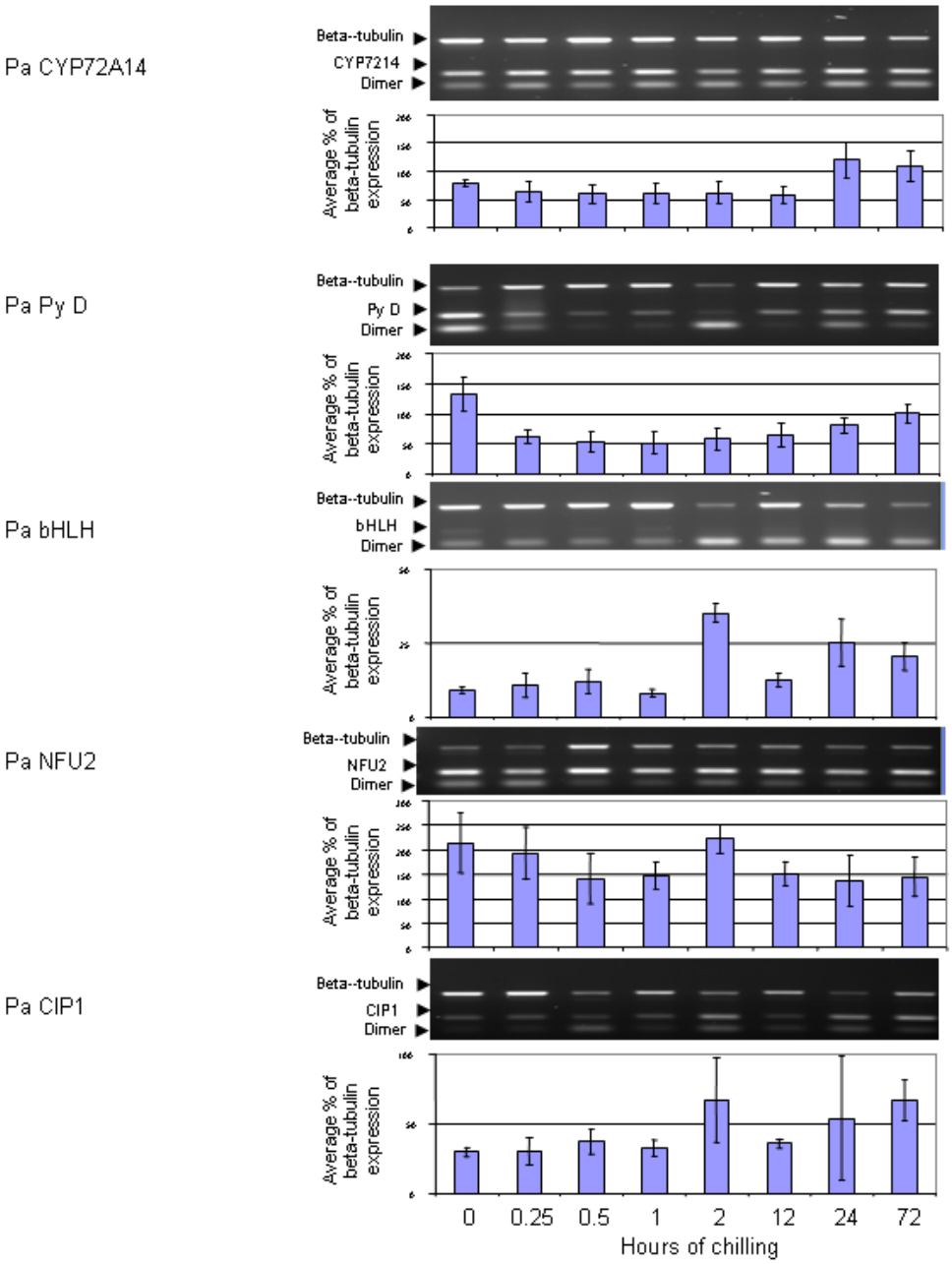

Figure 5.2. RT-PCR analysis of expression patterns of five *P. angustissimus* genes. RT-PCR analysis was carried out for each gene using RNA extracted from tissue of plants harvested at 0, 0.25, 0.5, 1, 2, 12, 24, and 72 hours of exposure to 7 ^{o}C day / 5 ^{o}C night. Changes in gene expression were estimated based on the expression of beta-tubulin, which was amplified in the same reaction (one example shown). Means and standard error for three biological replicates presented. CYP7214, cytochrome P450; Py D, pyruvate decarboxylase; bHLH, basic helix-loop-helix; NFU2; CIP1, COP (constitutive photomorphogenic) interactive protein. Gel images are of one biological replicate shown.

time-point than the non-chilled control in two of the three biological replicates. The coefficient of variance (CV) for the *P. vulgaris* HSP83 and GABAr RT-PCR experiments was 42.3 and 43.0, respectively.

A *P. vulgaris* gene putatively coding for an abscisic acid activated protein (ABAp) was not up- or down-regulated significantly at any of the time-points sampled compared to non-chilled control levels (Figure 5.1). In contrast, this gene appeared to be up-regulated using hybridizations of cDNA from 72-hour chilled *P. vulgaris* plants compared to hybridizations of cDNA from non-chilled *P. vulgaris* plants using the interspecific macroarray. The CV for the *P. vulgaris* ABAp RT-PCR experiments was 84.8, as a result of one of the biological replicates behaving differently from the other two. In two of the three biological replicates used in the RT-PCR experiments, ABAp was shown to be up-regulated at the 72 hour time point compared to the expression of this gene in non-chilled control plants (0 time-point). This gene was also shown to be down-regulated during the first two hours of chilling, and a recovery was seen in expression at the 12 hour time-point after the drop.

The expression patterns of a gene putatively coding for a cytochrome P450 protein (CYP72A14) in both *P. vulgaris* and *P. angustissimus* was surveyed in both species using RT-PCR. In *P. vulgaris*, this gene was down-regulated within the first 15 minutes of chilling and did not recover to non-chilled levels of expression until the 12 hour chilling time-point, and was once again down-regulated at the 72 hour time-point to where the expression level was lower than that for both the non-chilled control and the 12 hour chilled sample expression levels (Figure 5.1). Based on the macroarray results, this gene also appeared to be down-regulated in *P. vulgaris* in hybridizations of

cDNA from 72-hour chilled *P. vulgaris* plants compared to hybridizations of cDNA from non-chilled *P. vulgaris* plants using the interspecific macroarray. In *P. angustissimus*, this same gene showed up-regulation in gene expression at the 24 hour time-point, although it did not show significant up-regulation at the 72-hour time-point compared to the non-chilled control time-point (Figure 5.2). The 72 hour time-point showed greater expression of cytochrome P450 than time-points 0.25, 0.5, 1, 2, and 12 hours of chilling in *P. angustissimus* RT-PCR experiments. This cytochrome P450 was found to be up-regulated based on the macroarray results in *P. angustissimus* using hybridizations of cDNA from 72-hour chilled *P. angustissimus* plants compared to hybridizations of cDNA from non-chilled *P. angustissimus* plants. In two of the three biological replicates of the RT-PCR experiments, this cytochrome P450 showed greater expression in the 72 hours of chilling sample than the non-chilled control sample. The CV for RT-PCR experiments for *P. vulgaris* cytochrome P450 was 47.6, and the CV for RT-PCR experiments for *P. angustissimus* cytochrome P450 was 39.5.

In *P. angustissimus*, a gene putatively coding for pyruvate decarboxylase isozyme 1 (PyD) showed down-regulation within 15 minutes of low temperature exposure, and did not recover to control levels of expression until the 24 hours of chilling time-point (Figure 5.2). The CV for the *P. angustissimus* PyD RT-PCR experiments was 42.8. This PyD was shown to be down-regulated in hybridizations of cDNA from 72-hour chilled *P. angustissimus* plants compared to hybridizations of cDNA from non-chilled *P. angustissimus* plants using the interspecific macroarray experiments, and in two of the three biological replicates of the RT-PCR experiments,

this gene was found to show lower levels of expression in the 72 hours of chilling time-point than the non-chilled control.

A gene putatively coding for a *P. angustissimus* basic helix-loop-helix (bHLH) transcription factor did not show significant up-regulation at the 72-hour chilled time-point compared to non-chilled control over the three biological replicates in the RT-PCR experiments (Figure 5.2). This gene displayed up-regulation in hybridizations of cDNA from 72-hour chilled *P. angustissimus* plants compared to hybridizations of cDNA from non-chilled *P. angustissimus* plants using the interspecific macroarray experiments. The CV for the *P. angustissimus* bHLH RT-PCR experiments was 43.1. This gene did show higher levels of expression in two of the three RT-PCR experiments when comparing the 72 hour chilling time-point to the non-chilled time-point in *P. angustissimus*. This bHLH gene also showed higher expression levels at 2 and 24 hour chilling time-points than time-points 0, 0.25, 0.5, 1, and 12 hours of chilling, although the levels was not significantly higher than that for the 72 hour chilling time-point.

An NFU2-type gene did not have significantly altered transcription levels over the duration of low temperature exposure sampled in *P. angustissimus*. This gene displayed down-regulation in hybridizations of cDNA from 72-hour chilled *P. angustissimus* plants compared to hybridizations of cDNA from non-chilled *P. angustissimus* plants using the interspecific macroarray. In each of the three biological replicates used in the RT-PCR experiments, this gene showed lower levels of expression in the 72 hour chilled time-point than the non-chilled control time-point. The CV for the *P. angustissimus* NFU2 RT-PCR experiments was 36.9.

A *P. angustissimus* gene coding for a constitutive photomorphogenic interactive protein, while not showing significant changes in expression across all biological replicates, showed greater transcript levels in the 72 hours of chilling time-point compared to non-chilled samples in two of three biological replicates. In stark contrast, this gene appeared to be down-regulated in hybridizations of cDNA from 72-hour chilled *P. angustissimus* plants compared to hybridizations of cDNA from non-chilled *P. angustissimus* plants using the interspecific macroarray experiments. The CV for the *P. angustissimus* constitutive photomorphogenic interactive protein 1 (CIP1) RT-PCR experiments was 70.7.

5.4 Discussion

In order to discern the relative expression patterns of genes of *P. vulgaris* and *P. angustissimus* at various durations of low temperature exposure, semi-quantitative RT-PCR experiments were performed. Genes were selected for RT-PCR experiments on the basis of their putative roles in low temperature response in *Phaseolus* and the availability of sequence information for the *P. vulgaris* and *P. angustissimus* homologues of the *M. sativa* cDNA sequences used in interspecific cDNA hybridizations. Gene expression levels were evaluated in comparison to beta-tubulin expression levels in the same time-points, as beta-tubulin has been successfully used as a reference in previous low temperature gene expression studies (Lee et al., 2001; Chinnusamy et al., 2003; Sharma et al., 2007). Selection of genes from macroarray experiments was limited to those which had *Phaseolus* cDNA sequence identity matches of >80% with the *M. sativa* cDNA sequences represented on the macroarray.

Specific transcript accumulation measurement is required to reliably quantify alterations in expression. One common method of evaluating transcript levels is the Northern blotting protocol, which has a variety of limitations, including the demand for copious quantities of RNA and a lengthy regimen. Semi-quantitative RT-PCR is a more sensitive method than Northern blotting for transcript detection in plant molecular biology. A critical step in avoiding bias in RT-PCR experiments is the measurement of an internal reference gene as a means of estimating comparable levels of mRNA. Reference genes that have been commonly used in RT-PCR experiments that show limited variation in expression include actin (Sturzenbaum and Kille, 2001), glyceraldehyde -3- phosphate dehydrogenase (Bezier et al., 2002) and beta-tubulin (Ozturk et al., 2002).

The results of the RT-PCR experiments were generally different from those observed in the interspecific macroarray experiments when comparing non-chilled and 72 hour chilled expression levels to the hybridization intensities detected on the macroarrays. Only one gene analyzed using RT-PCR showed expression levels that was generally consistent with the macroarray results; a *P. vulgaris* cytochrome P450 showed down-regulation in both the macroarray and significant down-regulation in RT-PCR experiments when comparing 0 and 72 hour time-points over three biological replicates. The other genes analyzed to confirm the macroarray results either showed no significant changes in gene expression levels in RT-PCR experiments when comparing the 0 and 72 hours of chilling time-points or, in the two of three biological replicates of *P. vulgaris* HSP83 and *P. angustissimus* CIP1, the opposite change in gene expression was observed. The use of a more sensitive method of measuring mRNA

levels, such as directly measuring nucleic acid copy numbers in real-time quantitative RT-PCR reactions may be warranted, as ethidium bromide stained agarose gels are only capable of detecting 10-fold changes whereas real-time quantitative techniques are capable of detecting 2-fold changes in DNA (see tutorial from Applied Biosystems 2007). Alternatively, crude visual scoring using Northern blotting protocols have been used extensively to confirm trends that may not be accurately reflected in semi-quantitative RT-PCR experiments. Also, the sequence similarity threshold used to identify homologues (80%) leaves the potential of selecting an inappropriate *Phaseolus* homologue. One also cannot rule out that the natural biological variation may be considerable in these genes under chilling conditions in these species. The CV values seen in many of these RT-PCR experiments is possibly a result of natural biological variation which may have existed in gene expression levels across the three biological replicates. This variation may have been too great to establish consistent results with statistical significance for these particular genes. It is also important to note that when comparing the results presented here with results seen in other species that total aerial tissue was used to derive cDNA templates, whereas other

Often, gene expression levels in some of the biological replicates of these semi-quantitative RT-PCR experiments were consistent with the results observed in the macroarray experiments. In the particular cases of *P. vulgaris* GABAr and *P. angustissimus* PyD expression, the expression levels of these genes, as detected by RT-PCR, were lower in two of the three biological replicates when comparing 72 hour chilled plants with non-chilled plants in a manner consistent with the results seen in the macroarray. Similarly, in the cases of *P. angustissimus* cytochrome P450 and bHLH,

the expression levels of these genes were higher in two of the three biological replicates when comparing 72 hour chilled plants with non-chilled plants in a manner consistent with the results seen in the macroarray. Thus, across five biological replicates (two assayed using interspecific macroarray hybridizations with *M. sativa*, and three using gene specific RT-PCR) these genes showed consistent trends in expression.

One of the strengths of RT-PCR experiments is their ability to give insight into the expression pattern of a given gene in each species over a time course of chilling temperature exposure, something not possible with the macroarray experiments without large quantities of RNA and multiple hybridizations. Consider the expression of cytochrome P450 in both *P. vulgaris* and *P. angustissimus*; both species appear to show a decrease in gene expression levels in the early minutes following exposure to chilling. *P. vulgaris* cytochrome P450 recovers transcript levels by 12 hours of chilling before transcript levels of this gene fall once again, as seen in the 24 and 72 hour treatments (Figure 5.1). In contrast, *P. angustissimus* cytochrome P450 gene expression levels not only recover later than *P. vulgaris* (24 hours of chilling in *P. angustissimus*, compared to 12 hours of chilling in *P. vulgaris*), but the transcript levels of this gene exceed control levels during the last 2 time-points. It is important to note here that the 12-hour time-point is the only time-point in which plants were experiencing subjective day, and expression levels of genes sampled at the 12-hour time-point should be interpreted in that context. Levels of cytochrome P450 expression in non-chilled plants appear to be maintained through hour 72 of chilling in *P. angustissimus*.

This cytochrome P450 is a member of a superfamily of monooxygenases, which are a group of heme-containing proteins that catalyze oxidative reactions in cells

(Narusaka et al., 2004). While this particular cytochrome P450 has yet to be functionally explored in plant stress, other members of the superfamily have been linked to abiotic stress via their transcriptional up-regulation in response to reactive oxygen species (ROS) and lipid metabolism in *Arabidopsis* (Narusaka et al., 2004). Down-regulation of these genes in *P. vulgaris*, as seen in section 4.3 and these RT-PCR experiments, is further indicative of the inability of that species to adjust properly to chilling temperatures, while the ability of *P. angustissimus* to not be damaged at chilling temperatures may be related to the maintenance of the expression levels of this gene. The down-regulation of these genes may responsible for the reduction of water content levels in *P. vulgaris* (see section 3.3), as a compromise in cytochrome P450 levels may have adverse effects on membrane lipid profiles.

Another interesting result of the time course RT-PCR experiments is the expression of the *P. vulgaris* GABA receptor, where the transcript levels of this gene were observed to spike at hour 24 of chilling. This may provide insight into the GABA signalling cycles of this species under this duration of chilling exposure. This pattern of expression may suggest that *P. vulgaris* is attempting to establish higher GABA signalling over the first 24 hours of chilling, but ultimately fails to sustain such levels, resulting in improper chilling stress resistance. In wheat, frost resistant cultivars showed high levels of GABA expression whereas frost-sensitive cultivars showed intermediate levels of expression (Mazzucotelli et al., 2006). It should be noted that the gene expression results presented here should be considered in the context that RNAs were derived from total aerial tissue samples, and not from leaf-only tissues as described in Mazzucotelli et al., 2006, and it is possible that RNAs of interest were

diluted with stem RNAs, which is important when interpreting the results of these experiments. In profiling the expression of GABA receptor, the results presented here provide insight into the potential roles of GABA as a signalling molecule in *P. vulgaris*.

NFU2s (Touraine et al., 2004) and CIPs (Matsui et al., 1995) have been shown to be involved in chloroplast biogenesis. Given that NFU2s are involved in chloroplast biogenesis (see Somerville, 1996; Huner et al., 1998; Allen and Ort, 2001) as well as its intermediate levels of expression seen in interspecific macroarray hybridizations (see section 4.3), this NFU2 gene was selected for monitoring over the time course of chilling in *P. angustissimus*. The CIP1 gene was selected for monitoring over the time course of chilling in *P. angustissimus* because of the observed down-regulation of this gene on the interspecific macroarray hybridizations (see section 4.3). Time course RT-PCR results confirmed the intermediate expression of this NFU2 gene in 72 hour chilled *P. angustissimus*, but showed that this CIP1 gene expression was shown to be up-regulated in 72 hour chilled *P. angustissimus*. Because representative amplicons of all RT-PCR experiments were sequenced, the results of the RT-PCR experiments are considered to be a more accurate measure of specific gene expression. Since both of these genes are involved in chloroplast biogenesis and both appear to show high levels of expression in the 72 hour chilling time point, these genes may be a part of the network of genes influencing the changes in levels of chlorophylls in *P. angustissimus* during low temperature exposure (see section 3.3).

In *P. angustissimus*, PyD gene expression levels dropped rapidly upon chilling exposure and gradually recovered transcript levels over the course of the chilling experiment yet never recovered transcript levels those seen in non-chilled plants. This

pattern of expression may suggest that *P. angustissimus* failed to maintain the expression of PyD under chilling conditions, but was striving to recover its expression over the course of chilling exposure. Pyruvate decarboxylases (PDC)s are known for catalyzing the first step in fermentation and have been shown to be up-regulated in response to anoxic conditions in *Arabidopsis* (Kursteiner et al., 2003). However, it appears that this experiment may be the first to measure the expression of this gene in response to low temperature conditions, as there are, as of this writing, no published studies of a time course of PDC expression in response to low temperature stress in plants.

A member of the bHLH transcription factor family was examined in *P. angustissimus* on the basis that it was strongly up-regulated in the macroarray experiments and that genes of this family have been implicated in low temperature response in other plant species. For instance, one member of the bHLH transcription factor family is encoded by *ICE1* (inducer of CBF3 (C-repeat binding factor 3) expression 1) (Zarka et al., 2003; Chinnusamy et al., 2003). CBF transcription factors have been shown to positively regulate *COR* (cold regulated) genes that are involved in cold acclimation and increased freezing tolerance in *Arabidopsis* and other plant species (Thomashow, 2001). Thus, the transcription of a bHLH gene in *P. angustissimus* as identified in macroarray experiments potentially indicates that a gene signalling cascade, possibly similar to the ICE-CBF-COR cascade in *Arabidopsis*, could be taking place to ameliorate damaging effects of chilling temperatures in this species. Results of time course RT-PCR experiments, however, show that this gene is not transcriptionally responsive to chilling in *P. angustissimus*. Chinnusamy et al. (2003) describe the

induction of *Arabidopsis* ICE1 expression in response to cold as "slight", which is consistent with the results of *P. angustissimus* bHLH in two of the three RT-PCR experiments described here, which possibly indicates that this bHLH is responding in a similar manner to the cold acclimation responsive *Arabidopsis* species. No corresponding bHLH was found in the *P. vulgaris* cDNA library, making a contrast study in the expression of this gene in the relatively more chilling sensitive member of *Phaseolus* impossible using RT-PCR, but some insight may be gained by noting that the macroarray results show that bHLH is down-regulated in *P. vulgaris*. It is not known at this time if the bHLH gene found in these array experiments is an ICE, and therefore cannot yet be compared to other ICE genes.

A gene coding for HSP83 showed little transcriptional modulation in the low temperature exposure durations sampled in *P. vulgaris* except for being up-regulated in 24 hour chilled plants. Heat shock proteins such as this have many functional properties, but the ability to serve as molecular chaperones is common to all heat shock proteins (Jakob et al., 1993; Schöffl et al., 1998). In protecting and stabilizing proteins from negative effects of stress, heat shock proteins are thought to play a role in chilling resistance (Guy et al., 1999) and freezing tolerance (Thomashow, 1999). Although HSP83 has yet to be comprehensively studied in low temperature response systems in plants, the inability of *P. vulgaris* to up-regulate this gene rapidly in response to low temperature exposure may be indicative of its inability to respond properly to chilling temperatures. Even when this HSP was up-regulated strongly in the 24 hour chilled *P. vulgaris* sample, these levels of gene regulation were not sustained in the 72 hour chilled sample, as the expression levels of this gene fell significantly from levels

observed in 24 hour chilled samples. Confounding these results are the results of the macroarray experiments where this gene was found to be up-regulated in 72 hour chilled *P. vulgaris* compared to non-chilled control samples. Once again, variation amongst biological replicates could potentially be an explanation as to why this gene did not show consistent results across biological replicates and technical approaches.

A *P. vulgaris* gene that showed expression levels that were among the lowest of those sampled was an abscisic acid activated protein (ABAp) that is similar to a family of aldo-keto reductases. Aldo-keto reductase proteins are thought to play a role in alleviating low temperature induced photooxidative stress (Lang et al., 2005) by detoxifying damaging reactive oxygen species (ROS; Hutin et al., 2003; Zhang et al., 2005). This gene, and other ABAps, can influence the physiological responses to low temperature exposure, such as membrane integrity and tissue dehydration. Since this gene does not appear to be significantly up-regulated in *P. vulgaris* under chilling conditions, it may be extrapolated that an effective ABA response is not occurring in this species and that low temperature induced photooxidative damage may be occurring unmediated.

In some cases, the *M. sativa* nucleic acid sequences for specific spots on the array were not of a sufficiently high quality to conduct productive BLAST searches of *Phaseolus* sequence databases, while in other cases, no suitable sequence homology was seen between quality *M. sativa* sequences and the *Phaseolus* cDNA library. Considering that the *M. sativa* cDNA library was derived from alfalfa crowns that were treated for two weeks at 2 ^{o}C day / -2 ^{o}C night, whereas the *Phaseolus* libraries were derived from aerial tissues of zero and three day chilled plants, one might expect that

not all of the same genes would show up in all cDNA libraries. Furthermore, despite both being legumes, alfalfa and common bean demonstrate great differences with regard to their respective ability to cope with low temperature stress. Alfalfa can survive to -16 ^{o}C after cold acclimation (Monroy et al., 1993) while both of these *Phaseolus* species are killed at -3.5 ^{o}C. An ideal situation would have been to have available sequence information in each *Phaseolus* species which corresponded to each *M. sativa* clone of interest, but these confounding factors, taken together, greatly reduced the types and numbers of genes available for assay in using RT-PCR.

Using time course RT-PCR approaches to compare and contrast gene expression in chilled *P. vulgaris* and *P. angustissimus*, it is clear that these two species respond to low temperatures differently. Comparing the results of RT-PCR experiments to results of macroarray experiments may indicate a large degree of biological variation within *Phaseolus* gene transcription in low temperatures. More work needs to be done in order to obtain the full scope of the impacts of the expression of these genes in low temperatures, but these initial experiments yielded much needed insight into gene expression over the early periods of chilling stress in *Phaseolus*. These efforts represents one of the first studies to use functional genomic approaches to examine low temperature stress of the *Phaseolus* genus, and results of this research gives insight into the gene expression responses of these species to low temperature exposure.

6. General discussion

The research presented here demonstrates many ways two species of bean, *Phaseolus vulgaris* L. and *Phaseolus angustissimus* L., respond to low temperatures. Attempts were made to estimate the ability of each species to survive freezing temperatures, which showed that *P. angustissimus* survived lower temperatures than *P. vulgaris*. Responses to chilling temperatures were quantified using plant height, water content, and chlorophyll content parameters, which illustrated that both species ceased normal growth rates, although *P. angustissimus* appeared to have greater ability to cope with low temperatures with regard to water and chlorophyll content. A visual comparison of the two species suggests that *P. angustissimus* is better than *P. vulgaris* at arresting its growth rate over prolonged chilling durations in addition to displaying less leaf discolouration. While arresting growth rate at low temperatures may be critical to a plant coping with low temperature stress, bean growers may be ideally looking for genotypes that will maintain normal growth rates in low temperatures so as to achieve maturity before fall frosts. However, *P. angustissimus* may serve as a good source of germplasm for drought tolerance and photosynthetic stability under stress conditions.

Gene expression profiling using a *Medicago sativa* cDNA array revealed that *P. vulgaris* had a comparably greater transcriptional response to chilling temperatures, and this transcriptional response is that of a general trend towards down-regulation. In contrast, *P. angustissimus* showed a general trend towards up-regulation using this

interspecific macroarray experiment. A subset of 40 *M. sativa* cDNAs specifically selected for analysis were not found to be responsive to the chilling conditions in either species, in general, as only three genes were found to be transcriptionally responsive in both species combined. This result indicates that neither species responds to low temperatures in a manner observed in other plant species that are responsive to low temperatures, i.e. in a way that helps them survive sub-zero temperatures. In the array experiments, *P. vulgaris* down-regulated genes that would assist them in coping with low temperature stress, including cytochrome P450s, which may indicate that this species is responding differently compared to tolerant plant species under similar stress conditions. Among the genes found to be up-regulated using the *M. sativa* cDNA array in *P. angustissimus* was a basic helix-loop-helix gene and two cytochrome P450 genes, while it down-regulated genes related to photosynthesis and energy metabolism. Since protecting plants from damage caused by increased ROS and desiccation are important to cope with low temperatures, these results indicate that *P. angustissimus* plants respond to low temperatures better than *P. vulgaris*

Results of semi-quantitative RT-PCR, while generally not reflecting the results of the array experiments, nonetheless revealed insight into the expression of selected genes expression over varying durations of chilling temperature exposure. One of the key findings of these experiments was that the expression of a gene coding for a cytochrome P450 protein dropped rapidly under chilling conditions in *P. vulgaris*, then returned to non-chilled expression levels at the 12 hour time-point of low temperature exposure, before expression levels dropped again by the 72 hours of chilling time-point. In contrast, *P. angustissimus* cytochrome P450 expression fell rapidly upon exposure to

chilling temperatures, then recovered to control levels of expression by the 24 hours of chilling time-point, and expression of this gene was equivalent to control levels of expression at the 72 hours of chilling time-point. These results highlight some of the dynamics of low temperature gene expression patterns in these *Phaseolus* species. .

Investigations of complex gene regulatory pathways are becoming increasingly crucial to understand plant responses to environmental stimuli. It is also critical to link gene expression responses to plant physiological responses. Reported here are some physiological and gene expression responses of these two bean species with regard to low temperature exposure. It is increasingly necessary to develop tools to conceptualize gene responses in species lacking well-developed genomic resources, such as the genus *Phaseolus*. In this research, cDNAs from cold acclimated *M. sativa* crowns were used to carry out such genomic studies, revealing valuable insight into changes in gene expression patterns in these bean species in response to low temperatures. While interspecific cDNA arrays reported here yielded compelling results, the difficulties encountered in conducting such research, both conceptually and procedurally, should not be overlooked. Genomic sequencing projects, which have already been completed in *Arabidopsis*, *Medicago truncatula,* and rice, need to be undertaken in *Phaseolus* in order to maximize the utilities of genomic approaches to studying this genus. Furthermore, genomics research should be compared to results of individual gene expression studies, such as RT-PCR, to obtain further insight into the dynamics of transcriptional responses. Identifying genes in this manner provides information to breeders seeking to positively select genes that may result in increases chilling tolerance and provides researchers insight into how bean species respond to chilling conditions.

For researchers, identifying and characterizing low temperature responsive genes in *Phaseolus* in this manner assists researchers in attaining a greater understanding of low temperature tolerance in bean.

As gene expression studies of this nature resolve pathways regarding stress responses, metabolic processes can begin to be understood. Gene expression studies, together with other physiology-based studies, will enable researchers to target key areas in modulating common bean physiological performance under low temperature conditions.

7. Conclusions and future work

The goal of this study was to compare and contrast the effects of low temperature exposure in *Phaseolus vulgaris* and *Phaseolus angustissimus*. The hypothesis of this experiment was that the wild bean species, *P. angustissimus*, survives better under low temperature exposure than the cultivated bean species, *P. vulgaris*, and that *P. vulgaris* and *P. angustissimus* would behave differently in chilled conditions both physiologically and with respect to gene regulation. A series of experiments were conducted to meet three major objectives to explore this hypothesis. Specifically, these objectives were to assess the ability to survive freezing, to quantify physiological changes in over various durations of low temperature exposure, and to identify and characterize changes in gene expression patterns in response to various durations of low temperature exposure.

While neither species increased in height when exposed to low temperature conditions, *P. vulgaris* showed tissue dehydration over the first three days of low temperature exposure compared to *P. angustissimus*. Also, *P. vulgaris* showed a decrease in total chlorophyll content on the third day of low temperature exposure whereas *P. angustissimus* did not. Interspecific macroarray experiments showed large differences in expression patterns between *P. vulgaris* and *P. angustissimus* regarding the genes sampled, and a number of key genes showed expression patterns over the first

three days of low temperature exposure that would seem to indicate that the wild bean species is responding better in low temperature conditions.

A potential for further exploration would be to evaluate the expression patterns of key genes identified in this array as more sequence information becomes available. Characterization of these genes would enable the development of molecular markers for low temperature tolerance in a *P. vulgaris* breeding program. From the study presented here, it appears that expression of cytochrome P450 is perhaps a strong candidate as a marker for greater low temperature tolerance, and thus exploration of variations of the promoter regions of this gene could be conducted to ensure selection of this cytochrome P450 expression in *P. vulgaris*. Promoter sequences are potential targets for selection of beneficial gene expression patterns that could lead to improvements in low temperature tolerance.

Based on the data presented here, future research can be conducted to better understand low temperature responses in these bean species. For instance, recent work in our group has identified another species of bean, *Phaseolus acutifolius*, which has been shown to survive lower temperatures than *P. angustissimus* (Martinez et al., 2007). Evaluating the responses of *P. acutifolius* with regard to its physiological responses to low temperature exposure would provide insight into the differences between this species of bean and those presented here. Also, considering that a large number of the *M. sativa* cDNA array membranes used in this experiment have been printed, a next step could be to hybridize cDNAs of non-chilled and chilled *P. acutifolius* using these macroarrays. Not only would this provide contrast to the hybridization studies of *P. vulgaris* and *P. angustissimus* provided here, but given the relatively greater potential

for *P. acutifolius* to cross with *P. vulgaris* than *P. angustissimus* (Gurusamy et al. 2006), the results of such experiments could be used to develop new indicators of low temperature tolerance and identify molecular markers in breeding programs aimed at producing a low temperature tolerant cultivated bean.

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9. Appendix

Medicago sativa clone ID	Normalized Average *P. vulgaris* (7/5 °C) / (23/18 °C)	Normalized Average *P. angustissimus* (7/5 °C) / (23/18°C)	Medicago sativa clone ID	Normalized Average *P. vulgaris* (7/5 °C) / (23/18°C)	Normalized Average *P. angustissimus* (7/5 °C) / (23/18°C)
Ms01_01a01	**4.89**	**0.70**	Ms03_22h06	**0.24**	**0.64**
Ms01_01a02	**0.36**	**0.85**	Ms03_22h07	**0.40**	**0.73**
Ms01_01a03	**0.18**	**0.85**	Ms03_22h08	**0.46**	**1.03**
Ms01_01a04	**0.11**	**1.49**	Ms03_22h09	**0.49**	**5.00**
Ms01_01a05	**0.17**	**1.07**	Ms03_22h10	**0.45**	**0.90**
Ms01_01a06	**0.17**	**1.81**	Ms03_22h11	**1.54**	**1.69**
Ms01_01a07	**0.21**	**1.52**	Ms03_22h12	**1.95**	**1.10**
Ms01_01a08	**0.38**	**1.07**	Ms03_23a01	**6.90**	**0.34**
Ms01_01a09	**0.19**	**1.23**	Ms03_23a02	**0.93**	**0.64**
Ms01_01a10	**0.02**	**1.49**	Ms03_23a03	**0.80**	**0.81**
Ms01_01a11	**0.92**	**4.99**	Ms03_23a04	**0.30**	**0.45**
Ms01_01a12	**0.02**	**1.26**	Ms03_23a05	**1.02**	**0.56**
Ms01_01b01	**0.50**	**2.33**	Ms03_23a06	**0.35**	**3.07**
Ms01_01b02	**1.63**	**0.57**	Ms03_23a07	**1.21**	**0.72**
Ms01_01b03	**0.07**	**0.94**	Ms03_23a08	**0.66**	**0.85**
Ms01_01b04	**0.54**	**0.87**	Ms03_23a09	**2.67**	**1.87**
Ms01_01b05	**11.28**	**0.66**	Ms03_23a10	**3.73**	**1.24**
Ms01_01b06	**1.61**	**0.88**	Ms03_23a11	**1.06**	**0.60**
Ms01_01b07	**0.73**	**2.23**	Ms03_23a12	**0.62**	**0.27**
Ms01_01b08	**0.29**	**0.42**	Ms03_23b01	**0.19**	**1.91**
Ms01_01b09	**1.65**	**1.39**	Ms03_23b02	**0.20**	**0.39**
Ms01_01b10	**0.44**	**1.80**	Ms03_23b03	**0.44**	**0.51**
Ms01_01b11	**0.32**	**0.53**	Ms03_23b04	**0.96**	**1.29**
Ms01_01b12	**0.90**	**1.09**	Ms03_23b05	**0.23**	**1.51**
Ms01_01c01	**0.17**	**0.38**	Ms03_23b06	**1.60**	**2.24**
Ms01_01c02	**0.62**	**0.36**	Ms03_23b07	**2.90**	**0.59**
Ms01_01c03	**0.33**	**2.60**	Ms03_23b08	**0.94**	**0.79**
Ms01_01c04	**0.25**	**0.73**	Ms03_23b09	**0.65**	**0.92**
Ms01_01c05	**0.19**	**0.50**	Ms03_23b10	**0.37**	**1.24**
Ms01_01c06	**1.86**	**1.26**	Ms03_23b11	**0.28**	**1.21**
Ms01_01c07	**0.52**	**1.10**	Ms03_23b12	**0.62**	**0.73**
Ms01_01c08	**0.03**	**1.89**	Ms03_23c01	**1.02**	**1.04**
Ms01_01c09	**0.35**	**0.76**	Ms03_23c02	**0.66**	**1.67**
Ms01_01c10	**0.12**	**0.77**	Ms03_23c03	**1.07**	**0.92**
Ms01_01c11	**0.48**	**1.15**	Ms03_23c04	**0.20**	**2.37**
Ms01_01c12	**0.20**	**1.19**	Ms03_23c05	**1.90**	**0.66**
Ms01_01d01	**0.15**	**1.51**	Ms03_23c06	**0.84**	**0.91**
Ms01_01d02	**0.40**	**3.05**	Ms03_23c07	**0.32**	**1.64**
Ms01_01d03	**0.25**	**0.93**	Ms03_23c08	**1.17**	**0.90**
Ms01_01d04	**3.43**	**0.57**	Ms03_23c09	**0.54**	**0.66**
Ms01_01d05	**0.77**	**1.09**	Ms03_23c10	**0.11**	**2.46**
Ms01_01d06	**11.98**	**1.25**	Ms03_23c11	**1.23**	**1.13**
Ms01_01d07	**3.16**	**0.71**	Ms03_23c12	**2.79**	**4.52**
Ms01_01d08	**0.62**	**0.41**	Ms03_23d01	**0.39**	**0.53**
Ms01_01d09	**1.00**	**1.83**	Ms03_23d02	**0.30**	**0.35**
Ms01_01d10	**0.03**	**0.94**	Ms03_23d03	**1.28**	**1.56**
Ms01_01d11	**0.27**	**0.89**	Ms03_23d04	**0.98**	**0.54**
Ms01_01d12	**0.41**	**1.35**	Ms03_23d05	**0.42**	**0.56**
Ms01_01e01	**0.43**	**1.05**	Ms03_23d06	**0.58**	**0.79**
Ms01_01e02	**0.37**	**0.91**	Ms03_23d07	**0.66**	**1.18**
Ms01_01e03	**0.56**	**1.07**	Ms03_23d08	**1.09**	**1.11**
Ms01_01e04	**0.29**	**1.19**	Ms03_23d09	**0.17**	**2.34**
Ms01_01e05	**4.38**	**1.06**	Ms03_23d10	**0.36**	**0.71**
Ms01_01e06	**0.45**	**1.01**	Ms03_23d11	**0.23**	**0.67**

Ms01_01e07	**0.83**	**0.69**	Ms03_23d12	**0.31**	**0.69**
Ms01_01e08	**1.91**	**0.74**	Ms03_23e01	**0.56**	**0.53**
Ms01_01e09	**0.77**	**0.54**	Ms03_23e02	**1.16**	**2.07**
Ms01_01e10	**0.40**	**0.47**	Ms03_23e03	**0.46**	**2.64**
Ms01_01e11	**0.33**	**0.28**	Ms03_23e04	**0.81**	**1.99**
Ms01_01e12	**1.21**	**2.32**	Ms03_23e05	**0.97**	**2.05**
Ms01_01f01	**0.31**	**1.03**	Ms03_23e06	**1.86**	**1.95**
Ms01_01f02	**0.10**	**0.75**	Ms03_23e07	**0.71**	**2.22**
Ms01_01f03	**0.63**	**2.03**	Ms03_23e08	**4.17**	**4.98**
Ms01_01f04	**0.27**	**0.66**	Ms03_23e09	**1.36**	**2.42**
Ms01_01f05	**1.22**	**0.97**	Ms03_23e10	**0.27**	**2.13**
Ms01_01f06	**0.68**	**0.79**	Ms03_23e11	**0.61**	**1.20**
Ms01_01f07	**0.10**	**1.70**	Ms03_23e12	**0.52**	**0.80**
Ms01_01f08	**0.80**	**0.91**	Ms03_23f01	**0.64**	**0.78**
Ms01_01f09	**0.03**	**0.75**	Ms03_23f02	**0.47**	**2.63**
Ms01_01f10	**0.21**	**1.10**	Ms03_23f03	**1.69**	**1.34**
Ms01_01f11	**0.28**	**0.10**	Ms03_23f04	**1.13**	**1.51**
Ms01_01f12	**0.12**	**0.46**	Ms03_23f05	**0.85**	**0.98**
Ms01_01g01	**0.92**	**0.92**	Ms03_23f06	**0.89**	**1.14**
Ms01_01g02	**0.06**	**0.95**	Ms03_23f07	**0.92**	**1.16**
Ms01_01g03	**0.58**	**1.03**	Ms03_23f08	**0.40**	**0.54**
Ms01_01g04	**0.45**	**0.96**	Ms03_23f09	**0.45**	**2.75**
Ms01_01g05	**4.39**	**0.72**	Ms03_23f10	**2.27**	**1.28**
Ms01_01g06	**3.83**	**0.90**	Ms03_23f11	**0.33**	**0.85**
Ms01_01g07	**0.01**	**0.48**	Ms03_23f12	**0.38**	**0.78**
Ms01_01g08	**0.64**	**0.90**	Ms03_23g01	**0.49**	**0.53**
Ms01_01g09	**1.40**	**0.83**	Ms03_23g02	**0.95**	**1.42**
Ms01_01g10	**0.19**	**0.69**	Ms03_23g03	**0.96**	**0.77**
Ms01_01g11	**0.31**	**0.60**	Ms03_23g04	**0.42**	**0.49**
Ms01_01g12	**0.02**	**1.37**	Ms03_23g05	**0.33**	**0.40**
Ms01_01h01	**0.18**	**2.41**	Ms03_23g06	**1.87**	**0.89**
Ms01_01h02	**0.77**	**1.61**	Ms03_23g07	**2.03**	**1.05**
Ms01_01h03	**0.50**	**1.38**	Ms03_23g08	**1.21**	**0.71**
Ms01_01h04	**0.73**	**0.94**	Ms03_23g09	**0.44**	**0.59**
Ms01_01h05	**0.66**	**0.70**	Ms03_23g10	**0.16**	**0.54**
Ms01_01h06	**1.21**	**1.00**	Ms03_23g11	**0.22**	**0.92**
Ms01_01h07	**0.22**	**0.81**	Ms03_23g12	**0.42**	**0.58**
Ms01_01h08	**0.07**	**0.41**	Ms03_23h01	**0.89**	**1.14**
Ms01_01h09	**0.26**	**0.71**	Ms03_23h02	**0.53**	**0.79**
Ms01_01h10	**0.46**	**1.16**	Ms03_23h03	**0.28**	**0.39**
Ms01_01h11	**0.26**	**0.95**	Ms03_23h04	**0.39**	**0.96**
Ms01_01h12	**0.02**	**0.66**	Ms03_23h05	**0.39**	**1.02**
Ms01_02a01	**0.59**	**0.79**	Ms03_23h06	**0.41**	**0.64**
Ms01_02a02	**0.22**	**0.45**	Ms03_23h07	**1.33**	**0.65**
Ms01_02a03	**0.06**	**5.12**	Ms03_23h08	**0.34**	**0.72**
Ms01_02a04	**0.11**	**1.47**	Ms03_23h09	**1.06**	**0.91**
Ms01_02a05	**6.55**	**1.20**	Ms03_23h10	**0.26**	**0.55**
Ms01_02a06	**2.75**	**1.26**	Ms03_23h11	**0.22**	**0.75**
Ms01_02a07	**9.96**	**2.03**	Ms03_23h12	**1.67**	**0.49**
Ms01_02a08	**0.68**	**0.97**	Ms03_24a01	**1.02**	**0.29**
Ms01_02a09	**1.37**	**1.04**	Ms03_24a02	**0.79**	**0.37**
Ms01_02a10	**1.43**	**2.97**	Ms03_24a03	**0.41**	**0.60**
Ms01_02a11	**1.06**	**2.89**	Ms03_24a04	**1.11**	**0.79**
Ms01_02a12	**0.88**	**0.83**	Ms03_24a05	**0.40**	**0.48**
Ms01_02b01	**4.05**	**0.43**	Ms03_24a06	**0.32**	**0.46**
Ms01_02b02	**0.38**	**0.78**	Ms03_24a07	**0.47**	**0.46**
Ms01_02b03	**0.08**	**0.62**	Ms03_24a08	**1.29**	**0.59**
Ms01_02b04	**6.41**	**1.18**	Ms03_24a09	**1.98**	**1.08**
Ms01_02b05	**0.50**	**0.87**	Ms03_24a10	**1.18**	**0.91**

Ms01_02b06	**0.17**	**1.52**	Ms03_24a11	**2.09**	**0.61**
Ms01_02b07	**0.81**	**2.26**	Ms03_24a12	**0.46**	**0.46**
Ms01_02b08	**0.40**	**1.01**	Ms03_24b01	**0.68**	**0.37**
Ms01_02b09	**2.43**	**1.39**	Ms03_24b02	**0.18**	**0.44**
Ms01_02b10	**1.32**	**2.24**	Ms03_24b03	**0.33**	**0.60**
Ms01_02b11	**2.81**	**2.31**	Ms03_24b04	**0.82**	**0.15**
Ms01_02b12	**1.67**	**1.38**	Ms03_24b05	**0.63**	**0.51**
Ms01_02c01	**0.10**	**1.13**	Ms03_24b06	**0.37**	**0.59**
Ms01_02c02	**0.23**	**0.93**	Ms03_24b07	**0.89**	**0.84**
Ms01_02c03	**0.08**	**1.35**	Ms03_24b08	**0.68**	**0.62**
Ms01_02c04	**0.24**	**1.56**	Ms03_24b09	**0.93**	**1.28**
Ms01_02c05	**0.33**	**0.95**	Ms03_24b10	**0.97**	**3.24**
Ms01_02c06	**0.10**	**1.03**	Ms03_24b11	**1.20**	**1.13**
Ms01_02c07	**0.12**	**1.09**	Ms03_24b12	**0.36**	**0.64**
Ms01_02c08	**2.56**	**0.47**	Ms03_24c01	**0.31**	**1.53**
Ms01_02c09	**4.70**	**1.79**	Ms03_24c02	**1.51**	**0.66**
Ms01_02c10	**1.84**	**1.95**	Ms03_24c03	**0.19**	**0.16**
Ms01_02c11	**1.35**	**0.87**	Ms03_24c04	**0.63**	**3.95**
Ms01_02c12	**1.23**	**1.27**	Ms03_24c05	**0.60**	**0.65**
Ms01_02d01	**0.25**	**0.67**	Ms03_24c06	**0.46**	**1.82**
Ms01_02d02	**0.08**	**0.26**	Ms03_24c07	**0.75**	**2.28**
Ms01_02d03	**0.44**	**0.68**	Ms03_24c08	**0.66**	**0.37**
Ms01_02d04	**0.38**	**1.95**	Ms03_24c09	**0.40**	**3.35**
Ms01_02d05	**0.33**	**0.80**	Ms03_24c10	**0.26**	**1.92**
Ms01_02d06	**0.51**	**0.66**	Ms03_24c11	**0.33**	**0.39**
Ms01_02d07	**0.29**	**0.81**	Ms03_24c12	**0.30**	**0.11**
Ms01_02d08	**0.52**	**1.45**	Ms03_24d01	**1.92**	**0.93**
Ms01_02d09	**1.23**	**0.84**	Ms03_24d02	**0.26**	**0.58**
Ms01_02d10	**1.11**	**0.73**	Ms03_24d03	**0.74**	**0.67**
Ms01_02d11	**1.19**	**5.38**	Ms03_24d04	**0.53**	**1.16**
Ms01_02d12	**0.70**	**0.63**	Ms03_24d05	**1.67**	**0.53**
Ms01_02e01	**0.32**	**0.77**	Ms03_24d06	**0.80**	**0.31**
Ms01_02e02	**0.11**	**1.43**	Ms03_24d07	**0.36**	**0.46**
Ms01_02e03	**1.72**	**0.45**	Ms03_24d08	**0.97**	**0.33**
Ms01_02e04	**0.31**	**1.01**	Ms03_24d09	**0.26**	**1.49**
Ms01_02e05	**0.71**	**1.43**	Ms03_24d10	**0.33**	**0.60**
Ms01_02e06	**1.71**	**1.30**	Ms03_24d11	**0.28**	**0.40**
Ms01_02e07	**1.09**	**1.06**	Ms03_24d12	**0.30**	**0.92**
Ms01_02e08	**0.35**	**2.36**	Ms03_24e01	**0.90**	**0.59**
Ms01_02e09	**0.22**	**1.83**	Ms03_24e02	**0.61**	**0.91**
Ms01_02e10	**3.37**	**1.14**	Ms03_24e03	**0.31**	**0.54**
Ms01_02e11	**1.07**	**0.59**	Ms03_24e04	**0.93**	**0.62**
Ms01_02e12	**0.82**	**0.91**	Ms03_24e05	**1.00**	**0.34**
Ms01_02f01	**0.26**	**1.17**	Ms03_24e06	**1.08**	**0.66**
Ms01_02f02	**0.67**	**1.25**	Ms03_24e07	**1.12**	**0.84**
Ms01_02f03	**0.82**	**1.71**	Ms03_24e08	**1.45**	**0.76**
Ms01_02f04	**2.13**	**1.00**	Ms03_24e09	**0.29**	**0.38**
Ms01_02f05	**0.59**	**5.08**	Ms03_24e10	**0.11**	**0.86**
Ms01_02f06	**0.42**	**3.02**	Ms03_24e11	**0.15**	**4.12**
Ms01_02f07	**1.11**	**0.56**	Ms03_24e12	**0.39**	**0.92**
Ms01_02f08	**0.07**	**0.73**	Ms03_24f01	**0.74**	**0.68**
Ms01_02f09	**0.30**	**3.20**	Ms03_24f02	**3.86**	**2.17**
Ms01_02f10	**2.74**	**1.24**	Ms03_24f03	**0.50**	**0.67**
Ms01_02f11	**4.30**	**1.89**	Ms03_24f04	**0.57**	**1.77**
Ms01_02f12	**0.63**	**1.51**	Ms03_24f05	**1.39**	**0.32**
Ms01_02g01	**0.22**	**3.27**	Ms03_24f06	**1.73**	**0.84**
Ms01_02g02	**2.11**	**0.89**	Ms03_24f07	**0.41**	**0.46**
Ms01_02g03	**1.93**	**1.10**	Ms03_24f08	**0.55**	**0.63**
Ms01_02g04	**0.21**	**0.81**	Ms03_24f09	**0.78**	**1.29**

Ms01_02g05	**0.91**	**4.97**	Ms03_24f10	**0.54**	**0.34**
Ms01_02g06	**0.10**	**0.78**	Ms03_24f11	**1.37**	**0.67**
Ms01_02g07	**0.57**	**0.93**	Ms03_24f12	**0.27**	**0.46**
Ms01_02g08	**0.40**	**2.58**	Ms03_24g01	**1.27**	**0.89**
Ms01_02g09	**2.11**	**2.01**	Ms03_24g02	**3.37**	**0.80**
Ms01_02g10	**0.52**	**0.70**	Ms03_24g03	**0.61**	**1.88**
Ms01_02g11	**1.31**	**1.16**	Ms03_24g04	**1.05**	**1.48**
Ms01_02g12	**0.91**	**0.80**	Ms03_24g05	**0.34**	**0.40**
Ms01_02h01	**3.14**	**1.66**	Ms03_24g06	**0.68**	**0.60**
Ms01_02h02	**0.09**	**1.34**	Ms03_24g07	**0.87**	**1.43**
Ms01_02h03	**0.94**	**0.99**	Ms03_24g08	**0.37**	**0.65**
Ms01_02h04	**0.15**	**1.23**	Ms03_24g09	**0.63**	**0.58**
Ms01_02h05	**1.01**	**1.09**	Ms03_24g10	**0.14**	**0.69**
Ms01_02h06	**0.94**	**0.78**	Ms03_24g11	**0.46**	**1.24**
Ms01_02h07	**0.35**	**1.01**	Ms03_24g12	**0.35**	**0.42**
Ms01_02h08	**0.09**	**1.17**	Ms03_24h01	**2.16**	**2.22**
Ms01_02h09	**1.56**	**0.66**	Ms03_24h02	**1.03**	**0.61**
Ms01_02h10	**1.46**	**0.80**	Ms03_24h03	**3.59**	**0.64**
Ms01_02h11	**2.11**	**0.67**	Ms03_24h04	**2.51**	**1.62**
Ms01_02h12	**2.08**	**0.67**	Ms03_24h05	**0.74**	**0.71**
Ms01_03a01	**0.50**	**0.52**	Ms03_24h06	**0.37**	**0.57**
Ms01_03a02	**0.99**	**0.97**	Ms03_24h07	**0.43**	**2.17**
Ms01_03a03	**3.87**	**0.60**	Ms03_24h08	**0.22**	**0.72**
Ms01_03a04	**1.29**	**0.70**	Ms03_24h09	**0.91**	**0.54**
Ms01_03a05	**1.82**	**0.85**	Ms03_24h10	**0.37**	**0.60**
Ms01_03a06	**2.55**	**1.37**	Ms03_24h11	**0.93**	**0.35**
Ms01_03a07	**2.22**	**0.65**	Ms03_24h12	**0.28**	**1.40**
Ms01_03a08	**0.19**	**0.87**	Ms03_25a01	**0.42**	**0.62**
Ms01_03a09	**0.12**	**0.93**	Ms03_25a02	**0.52**	**0.42**
Ms01_03a10	**0.70**	**3.26**	Ms03_25a03	**4.89**	**1.49**
Ms01_03a11	**0.44**	**1.99**	Ms03_25a04	**1.51**	**0.78**
Ms01_03a12	**0.81**	**1.27**	Ms03_25a05	**0.94**	**0.72**
Ms01_03b01	**1.00**	**3.06**	Ms03_25a06	**0.92**	**1.52**
Ms01_03b02	**1.45**	**0.54**	Ms03_25a07	**0.79**	**0.62**
Ms01_03b03	**0.57**	**0.38**	Ms03_25a08	**0.37**	**1.60**
Ms01_03b04	**2.51**	**0.94**	Ms03_25a09	**2.93**	**1.86**
Ms01_03b05	**1.39**	**1.80**	Ms03_25a10	**1.18**	**1.20**
Ms01_03b06	**0.14**	**0.79**	Ms03_25a11	**1.47**	**1.41**
Ms01_03b07	**2.05**	**1.38**	Ms03_25a12	**1.13**	**0.84**
Ms01_03b08	**0.25**	**1.33**	Ms03_25b01	**0.56**	**0.48**
Ms01_03b09	**1.28**	**2.76**	Ms03_25b02	**0.60**	**0.29**
Ms01_03b10	**0.56**	**2.83**	Ms03_25b03	**0.33**	**0.25**
Ms01_03b11	**0.19**	**5.08**	Ms03_25b04	**0.51**	**0.45**
Ms01_03b12	**0.25**	**2.01**	Ms03_25b05	**0.88**	**0.44**
Ms01_03c01	**1.50**	**0.47**	Ms03_25b06	**0.73**	**0.72**
Ms01_03c02	**1.35**	**0.33**	Ms03_25b07	**0.25**	**0.56**
Ms01_03c03	**0.33**	**0.58**	Ms03_25b08	**0.40**	**0.83**
Ms01_03c04	**1.79**	**0.70**	Ms03_25b09	**0.13**	**1.43**
Ms01_03c05	**2.51**	**1.07**	Ms03_25b10	**0.62**	**0.74**
Ms01_03c06	**0.22**	**2.14**	Ms03_25b11	**0.29**	**3.12**
Ms01_03c07	**0.15**	**1.73**	Ms03_25b12	**0.38**	**0.43**
Ms01_03c08	**10.56**	**1.82**	Ms03_25c01	**0.16**	**0.35**
Ms01_03c09	**0.09**	**2.15**	Ms03_25c02	**0.97**	**0.71**
Ms01_03c10	**0.97**	**0.95**	Ms03_25c03	**0.14**	**0.73**
Ms01_03c11	**0.50**	**3.09**	Ms03_25c04	**0.34**	**1.01**
Ms01_03c12	**0.12**	**1.25**	Ms03_25c05	**1.02**	**0.93**
Ms01_03d01	**1.56**	**0.85**	Ms03_25c06	**0.26**	**0.41**
Ms01_03d02	**0.62**	**0.40**	Ms03_25c07	**0.44**	**0.51**
Ms01_03d03	**1.81**	**1.86**	Ms03_25c08	**0.62**	**0.75**

Ms01_03d04	**1.86**	**0.86**	Ms03_25c09	**0.17**	**0.24**
Ms01_03d05	**0.50**	**2.80**	Ms03_25c10	**0.96**	**0.58**
Ms01_03d06	**0.52**	**0.70**	Ms03_25c11	**0.16**	**0.46**
Ms01_03d07	**1.30**	**0.49**	Ms03_25c12	**0.36**	**0.08**
Ms01_03d08	**0.52**	**0.51**	Ms03_25d01	**0.16**	**0.67**
Ms01_03d09	**0.62**	**13.09**	Ms03_25d02	**0.38**	**0.52**
Ms01_03d10	**0.30**	**1.00**	Ms03_25d03	**0.60**	**1.17**
Ms01_03d11	**0.90**	**1.16**	Ms03_25d04	**1.51**	**1.00**
Ms01_03d12	**0.06**	**3.87**	Ms03_25d05	**0.58**	**1.34**
Ms01_03e01	**2.91**	**0.73**	Ms03_25d06	**0.25**	**0.53**
Ms01_03e02	**0.21**	**0.85**	Ms03_25d07	**0.65**	**0.37**
Ms01_03e03	**0.35**	**11.13**	Ms03_25d08	**2.41**	**0.49**
Ms01_03e04	**1.51**	**0.62**	Ms03_25d09	**1.00**	**1.34**
Ms01_03e05	**0.97**	**1.40**	Ms03_25d10	**0.30**	**0.34**
Ms01_03e06	**1.33**	**0.88**	Ms03_25d11	**0.64**	**1.17**
Ms01_03e07	**0.08**	**0.61**	Ms03_25d12	**0.19**	**0.34**
Ms01_03e08	**1.29**	**1.47**	Ms03_25e01	**0.50**	**1.05**
Ms01_03e09	**1.74**	**1.23**	Ms03_25e02	**0.42**	**1.63**
Ms01_03e10	**1.00**	**0.86**	Ms03_25e03	**0.13**	**2.60**
Ms01_03e11	**0.18**	**1.25**	Ms03_25e04	**0.32**	**0.73**
Ms01_03e12	**2.28**	**2.00**	Ms03_25e05	**0.36**	**0.80**
Ms01_03f01	**0.25**	**0.62**	Ms03_25e06	**0.35**	**0.94**
Ms01_03f02	**3.19**	**1.71**	Ms03_25e07	**0.93**	**0.98**
Ms01_03f03	**1.53**	**1.55**	Ms03_25e08	**0.44**	**0.76**
Ms01_03f04	**0.72**	**3.16**	Ms03_25e09	**0.33**	**0.86**
Ms01_03f05	**14.27**	**1.79**	Ms03_25e10	**1.60**	**0.95**
Ms01_03f06	**2.87**	**1.20**	Ms03_25e11	**0.29**	**1.79**
Ms01_03f07	**1.00**	**3.74**	Ms03_25e12	**0.24**	**0.36**
Ms01_03f08	**0.35**	**0.96**	Ms03_25f01	**1.16**	**0.23**
Ms01_03f09	**0.63**	**1.11**	Ms03_25f02	**2.23**	**1.61**
Ms01_03f10	**1.21**	**1.25**	Ms03_25f03	**0.40**	**2.03**
Ms01_03f11	**2.37**	**1.54**	Ms03_25f04	**1.78**	**0.92**
Ms01_03f12	**0.17**	**1.50**	Ms03_25f05	**0.70**	**0.56**
Ms01_03g01	**0.76**	**1.52**	Ms03_25f06	**0.87**	**0.68**
Ms01_03g02	**1.39**	**1.45**	Ms03_25f07	**0.34**	**0.57**
Ms01_03g03	**0.10**	**0.54**	Ms03_25f08	**1.72**	**0.29**
Ms01_03g04	**0.45**	**0.70**	Ms03_25f09	**0.42**	**1.05**
Ms01_03g05	**2.10**	**0.71**	Ms03_25f10	**0.70**	**0.84**
Ms01_03g06	**1.05**	**0.66**	Ms03_25f11	**0.31**	**1.84**
Ms01_03g07	**1.95**	**1.07**	Ms03_25f12	**0.27**	**0.48**
Ms01_03g08	**0.98**	**0.58**	Ms03_25g01	**0.44**	**1.31**
Ms01_03g09	**1.49**	**0.97**	Ms03_25g02	**2.72**	**4.75**
Ms01_03g10	**1.56**	**0.95**	Ms03_25g03	**1.58**	**1.43**
Ms01_03g11	**0.81**	**1.55**	Ms03_25g04	**0.59**	**0.60**
Ms01_03g12	**0.13**	**0.96**	Ms03_25g05	**2.29**	**1.28**
Ms01_03h01	**0.85**	**1.04**	Ms03_25g06	**0.64**	**1.14**
Ms01_03h02	**0.22**	**0.69**	Ms03_25g07	**0.49**	**1.16**
Ms01_03h03	**1.49**	**1.54**	Ms03_25g08	**1.86**	**0.63**
Ms01_03h04	**2.23**	**0.53**	Ms03_25g09	**2.54**	**0.97**
Ms01_03h05	**0.62**	**0.69**	Ms03_25g10	**0.26**	**0.91**
Ms01_03h06	**0.30**	**0.62**	Ms03_25g11	**0.38**	**0.47**
Ms01_03h07	**0.41**	**0.88**	Ms03_25g12	**0.26**	**0.56**
Ms01_03h08	**0.24**	**0.66**	Ms03_25h01	**2.57**	**1.36**
Ms01_03h09	**0.70**	**1.26**	Ms03_25h02	**0.35**	**0.68**
Ms01_03h10	**0.91**	**0.22**	Ms03_25h03	**0.43**	**2.25**
Ms01_03h11	**1.84**	**1.29**	Ms03_25h04	**0.97**	**0.44**
Ms01_03h12	**0.92**	**2.01**	Ms03_25h05	**0.37**	**0.57**
Ms01_04a01	**0.16**	**0.91**	Ms03_25h06	**0.23**	**0.17**
Ms01_04a02	**0.48**	**0.31**	Ms03_25h07	**0.19**	**0.62**

Ms01_04a03	**0.42**	**2.76**	Ms03_25h08	**1.78**	**0.87**
Ms01_04a04	**2.19**	**1.36**	Ms03_25h09	**1.46**	**0.80**
Ms01_04a05	**1.41**	**1.99**	Ms03_25h10	**2.05**	**0.53**
Ms01_04a06	**0.83**	**0.72**	Ms03_25h11	**0.56**	**0.74**
Ms01_04a07	**0.12**	**2.90**	Ms03_25h12	**0.26**	**0.62**
Ms01_04a08	**0.33**	**1.89**	Ms03_26a01	**0.58**	**0.20**
Ms01_04a09	**7.16**	**2.29**	Ms03_26a02	**0.26**	**0.90**
Ms01_04a10	**3.06**	**4.89**	Ms03_26a03	**0.31**	**0.50**
Ms01_04a11	**3.12**	**2.95**	Ms03_26a04	**0.75**	**7.64**
Ms01_04a12	**4.32**	**1.42**	Ms03_26a05	**0.74**	**2.20**
Ms01_04b01	**0.72**	**0.68**	Ms03_26a06	**0.98**	**1.21**
Ms01_04b02	**1.09**	**1.05**	Ms03_26a07	**0.26**	**0.93**
Ms01_04b03	**2.03**	**0.68**	Ms03_26a08	**0.53**	**1.06**
Ms01_04b04	**1.31**	**21.61**	Ms03_26a09	**2.06**	**5.28**
Ms01_04b05	**0.70**	**2.31**	Ms03_26a10	**0.85**	**6.66**
Ms01_04b06	**0.38**	**1.27**	Ms03_26a11	**0.76**	**1.58**
Ms01_04b07	**0.38**	**1.33**	Ms03_26a12	**0.40**	**2.68**
Ms01_04b08	**0.13**	**2.09**	Ms03_26b01	**0.41**	**0.38**
Ms01_04b09	**1.02**	**1.52**	Ms03_26b02	**0.32**	**0.69**
Ms01_04b10	**0.87**	**2.70**	Ms03_26b03	**0.31**	**2.33**
Ms01_04b11	**0.49**	**2.24**	Ms03_26b04	**0.55**	**0.79**
Ms01_04b12	**0.19**	**2.04**	Ms03_26b05	**1.65**	**1.21**
Ms01_04c01	**1.57**	**0.49**	Ms03_26b06	**0.42**	**0.60**
Ms01_04c02	**1.19**	**1.20**	Ms03_26b07	**0.89**	**1.22**
Ms01_04c03	**1.77**	**0.91**	Ms03_26b08	**0.40**	**0.72**
Ms01_04c04	**0.91**	**0.45**	Ms03_26b09	**0.09**	**1.35**
Ms01_04c05	**1.14**	**1.30**	Ms03_26b10	**0.21**	**1.80**
Ms01_04c06	**0.48**	**1.78**	Ms03_26b11	**0.70**	**0.46**
Ms01_04c07	**0.92**	**0.63**	Ms03_26b12	**1.58**	**0.90**
Ms01_04c08	**0.68**	**0.60**	Ms03_26c01	**0.26**	**1.09**
Ms01_04c09	**1.62**	**1.70**	Ms03_26c02	**0.72**	**0.92**
Ms01_04c10	**1.08**	**1.47**	Ms03_26c03	**0.22**	**0.59**
Ms01_04c11	**0.70**	**1.60**	Ms03_26c04	**0.19**	**7.01**
Ms01_04c12	**1.48**	**1.74**	Ms03_26c05	**0.50**	**0.69**
Ms01_04d01	**0.95**	**1.01**	Ms03_26c06	**0.70**	**0.53**
Ms01_04d02	**0.27**	**1.09**	Ms03_26c07	**0.59**	**0.28**
Ms01_04d03	**0.22**	**0.92**	Ms03_26c08	**1.00**	**0.71**
Ms01_04d04	**0.97**	**1.15**	Ms03_26c09	**3.39**	**1.08**
Ms01_04d05	**0.35**	**2.24**	Ms03_26c10	**1.38**	**1.39**
Ms01_04d06	**0.16**	**1.30**	Ms03_26c11	**1.34**	**0.96**
Ms01_04d07	**0.35**	**2.00**	Ms03_26c12	**0.56**	**0.84**
Ms01_04d08	**0.94**	**2.00**	Ms03_26d01	**0.29**	**0.98**
Ms01_04d09	**2.25**	**0.69**	Ms03_26d02	**1.17**	**0.75**
Ms01_04d10	**1.38**	**0.65**	Ms03_26d03	**0.29**	**1.17**
Ms01_04d11	**0.48**	**0.79**	Ms03_26d04	**0.37**	**1.71**
Ms01_04d12	**0.45**	**0.97**	Ms03_26d05	**0.21**	**0.23**
Ms01_04e01	**0.33**	**0.68**	Ms03_26d06	**0.22**	**0.49**
Ms01_04e02	**0.26**	**0.94**	Ms03_26d07	**0.99**	**0.59**
Ms01_04e03	**0.06**	**2.18**	Ms03_26d08	**1.09**	**0.67**
Ms01_04e04	**0.82**	**3.66**	Ms03_26d09	**1.87**	**0.85**
Ms01_04e05	**0.13**	**0.92**	Ms03_26d10	**0.38**	**0.64**
Ms01_04e06	**1.43**	**1.18**	Ms03_26d11	**0.74**	**0.85**
Ms01_04e07	**0.39**	**1.00**	Ms03_26d12	**0.25**	**0.57**
Ms01_04e08	**1.03**	**0.65**	Ms03_26e01	**0.67**	**0.67**
Ms01_04e09	**0.55**	**1.20**	Ms03_26e02	**0.30**	**0.82**
Ms01_04e10	**2.76**	**0.73**	Ms03_26e03	**1.35**	**0.70**
Ms01_04e11	**0.55**	**0.85**	Ms03_26e04	**0.33**	**0.65**
Ms01_04e12	**0.11**	**3.01**	Ms03_26e05	**0.34**	**0.28**
Ms01_04f01	**1.06**	**0.58**	Ms03_26e06	**0.63**	**0.84**

Ms01_04f02	**0.71**	**1.32**	Ms03_26e07	**0.89**	**0.70**
Ms01_04f03	**1.14**	**0.99**	Ms03_26e08	**0.42**	**0.63**
Ms01_04f04	**1.54**	**0.53**	Ms03_26e09	**0.20**	**0.44**
Ms01_04f05	**0.35**	**1.36**	Ms03_26e10	**0.12**	**2.44**
Ms01_04f06	**0.50**	**1.61**	Ms03_26e11	**0.26**	**0.77**
Ms01_04f07	**0.40**	**1.00**	Ms03_26e12	**0.20**	**1.20**
Ms01_04f08	**2.05**	**1.26**	Ms03_26f01	**0.46**	**1.00**
Ms01_04f09	**1.31**	**0.94**	Ms03_26f02	**0.84**	**2.88**
Ms01_04f10	**0.79**	**1.25**	Ms03_26f03	**0.60**	**0.76**
Ms01_04f11	**2.51**	**1.05**	Ms03_26f04	**0.57**	**2.82**
Ms01_04f12	**1.02**	**0.93**	Ms03_26f05	**0.46**	**0.64**
Ms01_04g01	**0.47**	**2.13**	Ms03_26f06	**0.47**	**0.56**
Ms01_04g02	**0.85**	**1.01**	Ms03_26f07	**0.31**	**0.53**
Ms01_04g03	**0.20**	**0.97**	Ms03_26f08	**0.76**	**0.36**
Ms01_04g04	**0.84**	**1.67**	Ms03_26f09	**1.26**	**0.43**
Ms01_04g05	**3.84**	**1.03**	Ms03_26f10	**1.78**	**0.44**
Ms01_04g06	**0.16**	**0.87**	Ms03_26f11	**0.58**	**0.83**
Ms01_04g07	**2.47**	**0.75**	Ms03_26f12	**0.40**	**0.36**
Ms01_04g08	**1.27**	**1.36**	Ms03_26g01	**0.30**	**1.67**
Ms01_04g09	**1.11**	**0.75**	Ms03_26g02	**1.55**	**0.43**
Ms01_04g10	**0.61**	**2.18**	Ms03_26g03	**4.17**	**0.28**
Ms01_04g11	**0.91**	**5.22**	Ms03_26g04	**0.73**	**0.35**
Ms01_04g12	**0.92**	**1.80**	Ms03_26g05	**2.22**	**1.50**
Ms01_04h01	**1.97**	**2.07**	Ms03_26g06	**0.31**	**0.93**
Ms01_04h02	**0.06**	**5.48**	Ms03_26g07	**0.23**	**0.83**
Ms01_04h03	**0.66**	**1.38**	Ms03_26g08	**2.53**	**1.37**
Ms01_04h04	**0.58**	**1.33**	Ms03_26g09	**3.15**	**3.71**
Ms01_04h05	**0.08**	**1.29**	Ms03_26g10	**0.33**	**0.14**
Ms01_04h06	**1.03**	**1.11**	Ms03_26g11	**0.34**	**0.43**
Ms01_04h07	**2.69**	**0.77**	Ms03_26g12	**0.18**	**0.79**
Ms01_04h08	**0.57**	**0.94**	Ms03_26h01	**0.89**	**2.16**
Ms01_04h09	**0.71**	**0.47**	Ms03_26h02	**0.19**	**0.47**
Ms01_04h10	**1.48**	**6.21**	Ms03_26h03	**0.21**	**1.20**
Ms01_04h11	**0.55**	**3.43**	Ms03_26h04	**0.38**	**1.91**
Ms01_04h12	**11.42**	**1.98**	Ms03_26h05	**0.28**	**0.39**
Ms01_05a01	**1.13**	**0.41**	Ms03_26h06	**0.52**	**0.36**
Ms01_05a02	**0.11**	**6.55**	Ms03_26h07	**0.10**	**0.51**
Ms01_05a03	**1.95**	**1.03**	Ms03_26h08	**0.33**	**0.41**
Ms01_05a04	**2.65**	**0.66**	Ms03_26h09	**0.73**	**0.64**
Ms01_05a05	**0.55**	**1.72**	Ms03_26h10	**0.13**	**1.56**
Ms01_05a06	**0.09**	**0.89**	Ms03_26h11	**1.22**	**0.41**
Ms01_05a07	**2.58**	**2.59**	Ms03_26h12	**0.41**	**0.45**
Ms01_05a08	**0.51**	**1.66**	Ms03_27a01	**0.27**	**1.09**
Ms01_05a09	**1.36**	**1.75**	Ms03_27a02	**0.47**	**0.56**
Ms01_05a10	**0.13**	**1.45**	Ms03_27a03	**1.54**	**1.26**
Ms01_05a11	**0.14**	**0.82**	Ms03_27a04	**0.59**	**0.50**
Ms01_05a12	**0.04**	**2.70**	Ms03_27a05	**0.58**	**0.55**
Ms01_05b01	**0.32**	**1.08**	Ms03_27a06	**2.76**	**1.69**
Ms01_05b02	**0.63**	**0.80**	Ms03_27a07	**0.61**	**0.40**
Ms01_05b03	**2.22**	**0.56**	Ms03_27a08	**0.27**	**0.92**
Ms01_05b04	**4.38**	**3.25**	Ms03_27a09	**1.46**	**1.08**
Ms01_05b05	**1.53**	**1.09**	Ms03_27a10	**0.84**	**0.64**
Ms01_05b06	**0.62**	**1.35**	Ms03_27a11	**1.01**	**0.66**
Ms01_05b07	**0.20**	**0.98**	Ms03_27a12	**0.31**	**0.36**
Ms01_05b08	**1.54**	**1.53**	Ms03_27b01	**0.96**	**1.13**
Ms01_05b09	**0.13**	**0.92**	Ms03_27b02	**0.33**	**0.34**
Ms01_05b10	**0.31**	**0.45**	Ms03_27b03	**0.47**	**0.67**
Ms01_05b11	**0.08**	**1.17**	Ms03_27b04	**0.86**	**0.47**
Ms01_05b12	**0.01**	**0.45**	Ms03_27b05	**2.18**	**0.60**

Ms01_05c01	**1.44**	**0.38**	Ms03_27b06	**2.19**	**0.67**
Ms01_05c02	**0.32**	**0.74**	Ms03_27b07	**0.90**	**1.66**
Ms01_05c03	**1.82**	**0.55**	Ms03_27b08	**2.33**	**0.44**
Ms01_05c04	**0.10**	**1.91**	Ms03_27b09	**0.38**	**0.88**
Ms01_05c05	**0.75**	**0.61**	Ms03_27b10	**1.68**	**0.63**
Ms01_05c06	**1.37**	**0.61**	Ms03_27b11	**0.71**	**0.61**
Ms01_05c07	**1.60**	**1.34**	Ms03_27b12	**0.62**	**1.20**
Ms01_05c08	**0.32**	**1.98**	Ms03_27c01	**0.44**	**0.74**
Ms01_05c09	**0.27**	**0.90**	Ms03_27c02	**0.46**	**0.45**
Ms01_05c10	**0.36**	**1.05**	Ms03_27c03	**5.02**	**0.58**
Ms01_05c11	**0.17**	**1.41**	Ms03_27c04	**1.09**	**0.86**
Ms01_05c12	**0.17**	**1.33**	Ms03_27c05	**1.14**	**0.39**
Ms01_05d01	**2.04**	**1.07**	Ms03_27c06	**0.79**	**0.60**
Ms01_05d02	**2.53**	**0.63**	Ms03_27c07	**1.55**	**1.41**
Ms01_05d03	**0.65**	**1.04**	Ms03_27c08	**0.55**	**1.34**
Ms01_05d04	**0.29**	**0.58**	Ms03_27c09	**0.53**	**0.79**
Ms01_05d05	**0.53**	**1.95**	Ms03_27c10	**1.16**	**0.38**
Ms01_05d06	**0.81**	**1.36**	Ms03_27c11	**0.49**	**1.45**
Ms01_05d07	**0.15**	**1.38**	Ms03_27c12	**0.66**	**1.57**
Ms01_05d08	**0.23**	**0.83**	Ms03_27d01	**0.21**	**0.91**
Ms01_05d09	**1.48**	**1.15**	Ms03_27d02	**1.44**	**1.56**
Ms01_05d10	**0.01**	**0.71**	Ms03_27d03	**1.78**	**0.74**
Ms01_05d11	**0.07**	**1.41**	Ms03_27d04	**1.23**	**0.48**
Ms01_05d12	**0.04**	**1.43**	Ms03_27d05	**1.21**	**0.41**
Ms01_05e01	**0.30**	**0.91**	Ms03_27d06	**1.18**	**0.60**
Ms01_05e02	**1.74**	**1.27**	Ms03_27d07	**0.94**	**0.75**
Ms01_05e03	**0.31**	**5.30**	Ms03_27d08	**1.01**	**0.54**
Ms01_05e04	**0.18**	**2.70**	Ms03_27d09	**3.09**	**5.99**
Ms01_05e05	**1.01**	**1.69**	Ms03_27d10	**0.29**	**0.99**
Ms01_05e06	**0.61**	**0.46**	Ms03_27d11	**0.48**	**0.44**
Ms01_05e07	**0.20**	**1.71**	Ms03_27d12	**0.57**	**0.66**
Ms01_05e08	**1.32**	**0.89**	Ms03_27e01	**0.86**	**1.38**
Ms01_05e09	**0.05**	**0.69**	Ms03_27e02	**2.23**	**0.91**
Ms01_05e10	**0.08**	**1.10**	Ms03_27e03	**0.97**	**3.76**
Ms01_05e11	**0.09**	**2.38**	Ms03_27e04	**1.01**	**0.56**
Ms01_05e12	**0.15**	**0.99**	Ms03_27e05	**0.65**	**0.37**
Ms01_05f01	**1.69**	**2.02**	Ms03_27e06	**0.80**	**0.89**
Ms01_05f02	**0.48**	**1.26**	Ms03_27e07	**1.41**	**4.72**
Ms01_05f03	**3.65**	**1.61**	Ms03_27e08	**0.47**	**0.78**
Ms01_05f04	**0.28**	**0.81**	Ms03_27e09	**0.24**	**0.37**
Ms01_05f05	**0.49**	**0.54**	Ms03_27e10	**0.83**	**0.37**
Ms01_05f06	**1.58**	**0.73**	Ms03_27e11	**0.21**	**0.51**
Ms01_05f07	**1.15**	**2.36**	Ms03_27e12	**0.22**	**0.69**
Ms01_05f08	**3.94**	**0.66**	Ms03_27f01	**1.25**	**1.02**
Ms01_05f09	**0.21**	**2.01**	Ms03_27f02	**2.91**	**0.93**
Ms01_05f10	**0.24**	**2.44**	Ms03_27f03	**0.59**	**0.55**
Ms01_05f11	**0.77**	**1.38**	Ms03_27f04	**2.01**	**0.44**
Ms01_05f12	**0.32**	**1.17**	Ms03_27f05	**2.12**	**0.79**
Ms01_05g01	**2.71**	**0.99**	Ms03_27f06	**1.20**	**0.58**
Ms01_05g02	**0.66**	**0.76**	Ms03_27f07	**0.71**	**0.72**
Ms01_05g03	**0.20**	**0.81**	Ms03_27f08	**3.40**	**0.32**
Ms01_05g04	**2.41**	**0.91**	Ms03_27f09	**0.25**	**0.31**
Ms01_05g05	**1.17**	**0.89**	Ms03_27f10	**0.53**	**0.53**
Ms01_05g06	**0.95**	**1.20**	Ms03_27f11	**2.15**	**0.91**
Ms01_05g07	**1.09**	**8.00**	Ms03_27f12	**0.51**	**1.09**
Ms01_05g08	**0.40**	**0.55**	Ms03_27g01	**0.91**	**0.50**
Ms01_05g09	**0.03**	**0.38**	Ms03_27g02	**2.11**	**0.88**
Ms01_05g10	**0.10**	**0.58**	Ms03_27g03	**1.24**	**0.74**
Ms01_05g11	**0.21**	**1.35**	Ms03_27g04	**0.64**	**0.27**

Ms01_05g12	**0.20**	**0.80**	Ms03_27g05	**3.01**	**0.31**
Ms01_05h01	**1.43**	**1.16**	Ms03_27g06	**0.98**	**0.57**
Ms01_05h02	**1.01**	**0.34**	Ms03_27g07	**0.49**	**0.88**
Ms01_05h03	**0.07**	**0.70**	Ms03_27g08	**1.26**	**0.32**
Ms01_05h04	**2.10**	**0.93**	Ms03_27g09	**1.25**	**0.42**
Ms01_05h05	**0.11**	**0.63**	Ms03_27g10	**0.19**	**0.84**
Ms01_05h06	**0.84**	**0.15**	Ms03_27g11	**0.34**	**0.80**
Ms01_05h07	**0.68**	**1.72**	Ms03_27g12	**0.32**	**0.57**
Ms01_05h08	**1.69**	**0.35**	Ms03_27h01	**0.51**	**0.67**
Ms01_05h09	**0.08**	**0.34**	Ms03_27h02	**0.28**	**0.47**
Ms01_05h10	**0.02**	**0.89**	Ms03_27h03	**0.33**	**0.42**
Ms01_05h11	**0.16**	**1.21**	Ms03_27h04	**0.99**	**0.67**
Ms01_05h12	**0.71**	**1.02**	Ms03_27h05	**0.44**	**0.66**
Ms02_01g06	**0.39**	**1.11**	Ms03_27h06	**0.45**	**0.36**
Ms02_04g02	**1.68**	**1.27**	Ms03_27h07	**0.83**	**1.82**
Ms02_08h02	**0.86**	**0.52**	Ms03_27h08	**1.81**	**0.25**
Ms02_10a10	**0.65**	**0.86**	Ms03_27h09	**1.93**	**5.58**
Ms02_10c11	**0.57**	**0.89**	Ms03_27h10	**0.71**	**0.88**
Ms02_10d07	**0.89**	**1.41**	Ms03_27h11	**0.70**	**4.53**
Ms02_12a12	**0.88**	**0.49**	Ms03_27h12	**0.13**	**0.26**
Ms02_13d11	**0.72**	**1.17**	Ms03_28a01	**0.07**	**1.38**
Ms02_15g08	**1.77**	**0.28**	Ms03_28a02	**0.78**	**1.29**
Ms03_06a10	**0.91**	**0.86**	Ms03_28a03	**1.12**	**1.79**
Ms03_06g07	**0.63**	**0.81**	Ms03_28a04	**0.52**	**0.57**
Ms03_07d08	**1.30**	**1.50**	Ms03_28a05	**1.64**	**2.35**
Ms03_11e08	**0.52**	**1.73**	Ms03_28a06	**1.04**	**0.52**
Ms03_11e11	**0.84**	**0.87**	Ms03_28a07	**2.52**	**1.58**
Ms03_12e09	**0.90**	**2.39**	Ms03_28a08	**0.98**	**1.41**
Ms03_14c04	**0.66**	**1.77**	Ms03_28a09	**1.73**	**2.61**
Ms03_14d12	**3.95**	**0.21**	Ms03_28a10	**1.38**	**1.27**
Ms03_15b04	**0.83**	**0.41**	Ms03_28a11	**0.78**	**2.31**
Ms03_19a01	**0.53**	**0.75**	Ms03_28a12	**15.15**	**0.97**
Ms03_19a02	**0.86**	**0.62**	Ms03_28b01	**0.26**	**10.00**
Ms03_19a03	**0.65**	**0.65**	Ms03_28b02	**0.10**	**0.43**
Ms03_19a04	**1.23**	**3.14**	Ms03_28b03	**1.55**	**0.72**
Ms03_19a05	**0.82**	**1.50**	Ms03_28b04	**2.11**	**0.38**
Ms03_19a06	**0.90**	**1.05**	Ms03_28b05	**3.29**	**23.70**
Ms03_19a07	**0.61**	**0.94**	Ms03_28b06	**3.08**	**3.54**
Ms03_19a08	**1.14**	**0.55**	Ms03_28b07	**2.85**	**0.86**
Ms03_19a09	**0.68**	**0.88**	Ms03_28b08	**1.45**	**2.58**
Ms03_19a10	**0.35**	**0.51**	Ms03_28b09	**0.35**	**1.76**
Ms03_19a11	**0.62**	**0.59**	Ms03_28b10	**0.92**	**16.86**
Ms03_19a12	**0.44**	**0.19**	Ms03_28b11	**1.16**	**2.39**
Ms03_19b01	**0.75**	**3.30**	Ms03_28b12	**1.44**	**1.41**
Ms03_19b02	**0.46**	**0.97**	Ms03_28c01	**1.26**	**0.78**
Ms03_19b03	**0.50**	**3.27**	Ms03_28c02	**0.10**	**0.97**
Ms03_19b04	**4.92**	**2.04**	Ms03_28c03	**0.74**	**1.53**
Ms03_19b05	**0.45**	**0.95**	Ms03_28c04	**0.18**	**0.50**
Ms03_19b06	**0.72**	**1.09**	Ms03_28c05	**0.89**	**9.12**
Ms03_19b07	**1.27**	**1.58**	Ms03_28c06	**2.20**	**1.09**
Ms03_19b08	**0.66**	**0.91**	Ms03_28c07	**0.87**	**3.88**
Ms03_19b09	**1.00**	**1.25**	Ms03_28c08	**2.20**	**5.44**
Ms03_19b10	**0.23**	**0.34**	Ms03_28c09	**3.52**	**1.04**
Ms03_19b11	**0.48**	**0.25**	Ms03_28c10	**0.69**	**2.16**
Ms03_19b12	**0.52**	**0.32**	Ms03_28c11	**1.71**	**2.30**
Ms03_19c01	**0.44**	**1.03**	Ms03_28c12	**1.05**	**4.64**
Ms03_19c02	**0.64**	**0.76**	Ms03_28d01	**0.59**	**0.83**
Ms03_19c03	**1.04**	**0.74**	Ms03_28d02	**0.79**	**0.96**
Ms03_19c04	**0.17**	**0.27**	Ms03_28d03	**1.94**	**6.99**

Ms03_19c05	**0.69**	**0.84**	Ms03_28d04	**0.10**	**0.72**
Ms03_19c06	**1.41**	**1.04**	Ms03_28d05	**0.70**	**0.40**
Ms03_19c07	**1.18**	**1.20**	Ms03_28d06	**2.04**	**0.87**
Ms03_19c08	**0.52**	**0.74**	Ms03_28d07	**0.57**	**2.40**
Ms03_19c09	**0.25**	**0.34**	Ms03_28d08	**3.41**	**0.67**
Ms03_19c10	**2.02**	**0.80**	Ms03_28d09	**0.24**	**1.44**
Ms03_19c11	**0.59**	**0.66**	Ms03_28d10	**1.05**	**2.47**
Ms03_19c12	**0.61**	**0.31**	Ms03_28d11	**0.65**	**1.59**
Ms03_19d01	**0.80**	**0.72**	Ms03_28d12	**1.50**	**1.42**
Ms03_19d02	**1.51**	**0.41**	Ms03_28e01	**1.51**	**1.04**
Ms03_19d03	**2.31**	**1.45**	Ms03_28e02	**1.53**	**1.40**
Ms03_19d04	**0.44**	**0.64**	Ms03_28e03	**0.66**	**4.65**
Ms03_19d05	**5.69**	**2.32**	Ms03_28e04	**0.87**	**0.64**
Ms03_19d06	**0.88**	**0.72**	Ms03_28e05	**0.66**	**1.27**
Ms03_19d07	**0.33**	**0.50**	Ms03_28e06	**0.54**	**1.24**
Ms03_19d08	**0.31**	**0.84**	Ms03_28e07	**2.58**	**1.88**
Ms03_19d09	**0.40**	**3.58**	Ms03_28e08	**0.49**	**3.11**
Ms03_19d10	**1.55**	**0.51**	Ms03_28e09	**0.76**	**1.19**
Ms03_19d11	**0.31**	**0.53**	Ms03_28e10	**0.22**	**0.97**
Ms03_19d12	**1.53**	**2.38**	Ms03_28e11	**1.10**	**1.58**
Ms03_19e01	**0.44**	**1.16**	Ms03_28e12	**0.96**	**1.72**
Ms03_19e02	**1.00**	**0.97**	Ms03_28f01	**2.72**	**3.33**
Ms03_19e03	**0.29**	**1.05**	Ms03_28f02	**2.45**	**2.60**
Ms03_19e04	**0.66**	**1.51**	Ms03_28f03	**0.72**	**1.80**
Ms03_19e05	**0.51**	**0.51**	Ms03_28f04	**0.81**	**0.85**
Ms03_19e06	**2.47**	**0.93**	Ms03_28f05	**0.80**	**2.56**
Ms03_19e07	**0.65**	**0.84**	Ms03_28f06	**1.64**	**1.53**
Ms03_19e08	**1.42**	**1.81**	Ms03_28f07	**0.74**	**3.22**
Ms03_19e09	**0.19**	**0.42**	Ms03_28f08	**2.24**	**0.90**
Ms03_19e10	**0.22**	**0.70**	Ms03_28f09	**1.82**	**3.52**
Ms03_19e11	**0.07**	**1.07**	Ms03_28f10	**0.37**	**2.33**
Ms03_19e12	**0.26**	**0.45**	Ms03_28f11	**1.63**	**1.39**
Ms03_19f01	**0.88**	**0.93**	Ms03_28f12	**1.80**	**1.08**
Ms03_19f02	**3.91**	**7.49**	Ms03_28g01	**0.24**	**3.92**
Ms03_19f03	**0.25**	**0.65**	Ms03_28g02	**0.39**	**2.50**
Ms03_19f04	**1.81**	**1.02**	Ms03_28g03	**1.38**	**2.87**
Ms03_19f05	**0.23**	**0.30**	Ms03_28g04	**1.04**	**2.13**
Ms03_19f06	**0.53**	**1.05**	Ms03_28g05	**0.09**	**1.34**
Ms03_19f07	**0.49**	**0.52**	Ms03_28g06	**0.24**	**2.85**
Ms03_19f08	**0.31**	**0.30**	Ms03_28g07	**3.36**	**0.82**
Ms03_19f09	**0.22**	**0.47**	Ms03_28g08	**0.98**	**1.73**
Ms03_19f10	**0.18**	**0.26**	Ms03_28g09	**0.31**	**2.98**
Ms03_19f11	**0.10**	**0.35**	Ms03_28g10	**1.00**	**2.75**
Ms03_19f12	**0.26**	**0.42**	Ms03_28g11	**1.12**	**2.71**
Ms03_19g01	**0.45**	**0.73**	Ms03_28g12	**1.06**	**1.17**
Ms03_19g02	**0.79**	**1.85**	Ms03_28h01	**0.37**	**0.25**
Ms03_19g03	**0.66**	**0.79**	Ms03_28h02	**0.43**	**0.90**
Ms03_19g04	**0.71**	**1.21**	Ms03_28h03	**1.43**	**0.63**
Ms03_19g05	**0.74**	**1.04**	Ms03_28h04	**0.26**	**1.04**
Ms03_19g06	**0.27**	**0.30**	Ms03_28h05	**0.73**	**1.00**
Ms03_19g07	**0.81**	**1.16**	Ms03_28h06	**0.21**	**1.89**
Ms03_19g08	**0.08**	**0.19**	Ms03_28h07	**0.44**	**1.25**
Ms03_19g09	**0.21**	**0.38**	Ms03_28h08	**1.70**	**1.21**
Ms03_19g10	**0.43**	**0.36**	Ms03_28h09	**0.88**	**1.29**
Ms03_19g11	**0.16**	**0.47**	Ms03_28h10	**0.47**	**17.38**
Ms03_19g12	**0.22**	**0.61**	Ms03_28h11	**2.08**	**3.41**
Ms03_19h01	**0.60**	**0.76**	Ms03_28h12	**2.56**	**2.35**
Ms03_19h02	**1.49**	**0.73**	Ms03_29a01	**2.82**	**1.31**
Ms03_19h03	**1.53**	**1.52**	Ms03_29a02	**0.84**	**1.73**

Ms03_19h04	**0.84**	**0.35**	Ms03_29a03	**0.82**	**0.72**
Ms03_19h05	**0.16**	**0.39**	Ms03_29a04	**0.32**	**0.43**
Ms03_19h06	**0.10**	**0.32**	Ms03_29a05	**0.34**	**1.34**
Ms03_19h07	**0.31**	**0.20**	Ms03_29a06	**0.38**	**2.58**
Ms03_19h08	**0.17**	**3.71**	Ms03_29a07	**2.61**	**0.89**
Ms03_19h09	**1.43**	**0.94**	Ms03_29a08	**3.10**	**2.22**
Ms03_19h10	**0.17**	**0.25**	Ms03_29a09	**0.18**	**3.97**
Ms03_19h11	**0.51**	**0.30**	Ms03_29a10	**2.65**	**7.01**
Ms03_19h12	**0.37**	**0.51**	Ms03_29a11	**1.10**	**1.72**
Ms03_20a01	**0.25**	**0.48**	Ms03_29a12	**1.69**	**2.79**
Ms03_20a02	**2.01**	**2.78**	Ms03_29b01	**0.09**	**1.08**
Ms03_20a03	**0.72**	**0.67**	Ms03_29b02	**0.66**	**0.47**
Ms03_20a04	**0.63**	**0.34**	Ms03_29b03	**1.24**	**3.58**
Ms03_20a05	**0.50**	**0.92**	Ms03_29b04	**0.72**	**1.04**
Ms03_20a06	**0.90**	**1.14**	Ms03_29b05	**0.42**	**0.52**
Ms03_20a07	**1.39**	**1.05**	Ms03_29b06	**5.53**	**7.23**
Ms03_20a08	**0.39**	**0.35**	Ms03_29b07	**2.49**	**2.32**
Ms03_20a09	**0.36**	**0.65**	Ms03_29b08	**2.05**	**1.32**
Ms03_20a10	**2.16**	**2.02**	Ms03_29b09	**0.34**	**9.08**
Ms03_20a11	**0.53**	**0.45**	Ms03_29b10	**1.06**	**3.73**
Ms03_20a12	**2.12**	**0.60**	Ms03_29b11	**3.39**	**0.93**
Ms03_20b01	**0.81**	**0.99**	Ms03_29b12	**0.56**	**1.94**
Ms03_20b02	**0.84**	**0.92**	Ms03_29c01	**0.09**	**1.39**
Ms03_20b03	**1.13**	**1.52**	Ms03_29c02	**1.78**	**0.96**
Ms03_20b04	**0.74**	**1.39**	Ms03_29c03	**0.36**	**0.27**
Ms03_20b05	**1.20**	**0.74**	Ms03_29c04	**1.62**	**0.77**
Ms03_20b06	**1.18**	**1.78**	Ms03_29c05	**0.21**	**3.27**
Ms03_20b07	**1.27**	**1.21**	Ms03_29c06	**1.75**	**6.81**
Ms03_20b08	**0.53**	**1.23**	Ms03_29c07	**0.99**	**7.30**
Ms03_20b09	**0.31**	**1.38**	Ms03_29c08	**0.53**	**1.17**
Ms03_20b10	**1.42**	**0.85**	Ms03_29c09	**0.88**	**21.17**
Ms03_20b11	**0.72**	**0.83**	Ms03_29c10	**1.21**	**2.38**
Ms03_20b12	**0.34**	**0.97**	Ms03_29c11	**1.39**	**3.56**
Ms03_20c01	**51.56**	**9.76**	Ms03_29c12	**0.44**	**0.55**
Ms03_20c02	**1.40**	**1.33**	Ms03_29d01	**0.26**	**0.55**
Ms03_20c03	**1.04**	**1.45**	Ms03_29d02	**0.62**	**0.39**
Ms03_20c04	**0.60**	**0.95**	Ms03_29d03	**2.92**	**1.37**
Ms03_20c05	**0.89**	**1.97**	Ms03_29d04	**0.95**	**3.38**
Ms03_20c06	**0.83**	**6.35**	Ms03_29d05	**1.59**	**0.39**
Ms03_20c07	**1.58**	**2.39**	Ms03_29d06	**1.18**	**0.96**
Ms03_20c08	**1.57**	**1.39**	Ms03_29d07	**1.65**	**1.03**
Ms03_20c09	**0.36**	**0.91**	Ms03_29d08	**2.41**	**4.85**
Ms03_20c10	**0.75**	**0.56**	Ms03_29d09	**0.27**	**0.62**
Ms03_20c11	**1.03**	**0.64**	Ms03_29d10	**1.04**	**2.29**
Ms03_20c12	**0.98**	**0.54**	Ms03_29d11	**2.23**	**1.66**
Ms03_20d01	**1.37**	**1.35**	Ms03_29d12	**0.14**	**8.87**
Ms03_20d02	**0.87**	**1.57**	Ms03_29e01	**0.53**	**2.37**
Ms03_20d03	**0.85**	**2.15**	Ms03_29e02	**5.33**	**0.60**
Ms03_20d04	**0.56**	**1.23**	Ms03_29e03	**1.60**	**1.96**
Ms03_20d05	**0.36**	**0.60**	Ms03_29e04	**2.73**	**0.72**
Ms03_20d06	**0.69**	**0.87**	Ms03_29e05	**1.06**	**0.59**
Ms03_20d07	**0.59**	**0.63**	Ms03_29e06	**0.21**	**0.44**
Ms03_20d08	**0.53**	**0.99**	Ms03_29e07	**0.53**	**7.92**
Ms03_20d09	**0.30**	**0.48**	Ms03_29e08	**0.26**	**2.00**
Ms03_20d10	**0.17**	**1.85**	Ms03_29e09	**1.07**	**3.61**
Ms03_20d11	**0.76**	**0.90**	Ms03_29e10	**0.51**	**1.96**
Ms03_20d12	**0.38**	**0.28**	Ms03_29e11	**0.74**	**6.42**
Ms03_20e01	**1.12**	**2.92**	Ms03_29e12	**0.67**	**1.43**
Ms03_20e02	**0.34**	**5.59**	Ms03_29f01	**3.20**	**2.61**

Ms03_20e03	**0.73**	**1.59**	Ms03_29f02	**3.33**	**1.50**
Ms03_20e04	**0.98**	**1.67**	Ms03_29f03	**1.03**	**1.87**
Ms03_20e05	**0.53**	**0.97**	Ms03_29f04	**0.41**	**1.77**
Ms03_20e06	**0.57**	**0.84**	Ms03_29f05	**1.47**	**0.80**
Ms03_20e07	**0.43**	**0.33**	Ms03_29f06	**1.63**	**0.38**
Ms03_20e08	**0.46**	**1.25**	Ms03_29f07	**0.78**	**1.05**
Ms03_20e09	**0.24**	**0.63**	Ms03_29f08	**4.29**	**10.88**
Ms03_20e10	**0.40**	**1.04**	Ms03_29f09	**1.50**	**1.44**
Ms03_20e11	**0.14**	**0.40**	Ms03_29f10	**0.56**	**1.50**
Ms03_20e12	**0.23**	**0.67**	Ms03_29f11	**0.29**	**0.58**
Ms03_20f01	**0.34**	**1.37**	Ms03_29f12	**0.61**	**16.55**
Ms03_20f02	**0.62**	**1.79**	Ms03_29g01	**0.59**	**2.64**
Ms03_20f03	**1.33**	**2.24**	Ms03_29g02	**2.62**	**0.76**
Ms03_20f04	**0.32**	**7.16**	Ms03_29g03	**0.88**	**8.95**
Ms03_20f05	**0.69**	**0.96**	Ms03_29g04	**1.22**	**0.83**
Ms03_20f06	**0.54**	**1.07**	Ms03_29g05	**2.05**	**0.69**
Ms03_20f07	**0.42**	**0.92**	Ms03_29g06	**1.52**	**0.84**
Ms03_20f08	**0.49**	**0.99**	Ms03_29g07	**0.60**	**0.65**
Ms03_20f09	**0.41**	**1.31**	Ms03_29g08	**1.12**	**1.25**
Ms03_20f10	**0.42**	**0.80**	Ms03_29g09	**0.57**	**0.93**
Ms03_20f11	**0.53**	**0.81**	Ms03_29g10	**1.52**	**15.86**
Ms03_20f12	**0.49**	**1.23**	Ms03_29g11	**0.36**	**3.43**
Ms03_20g01	**0.58**	**1.02**	Ms03_29g12	**0.45**	**4.12**
Ms03_20g02	**1.03**	**1.46**	Ms03_29h01	**0.40**	**14.48**
Ms03_20g03	**0.59**	**0.81**	Ms03_29h02	**1.61**	**1.49**
Ms03_20g04	**0.52**	**0.72**	Ms03_29h03	**0.51**	**6.36**
Ms03_20g05	**0.38**	**0.66**	Ms03_29h04	**1.13**	**0.35**
Ms03_20g06	**0.27**	**0.41**	Ms03_29h05	**3.49**	**0.48**
Ms03_20g07	**0.88**	**1.26**	Ms03_29h06	**0.43**	**0.92**
Ms03_20g08	**0.69**	**0.78**	Ms03_29h07	**0.90**	**3.77**
Ms03_20g09	**0.13**	**1.01**	Ms03_29h08	**0.24**	**0.48**
Ms03_20g10	**0.71**	**0.61**	Ms03_29h09	**0.18**	**0.40**
Ms03_20g11	**0.43**	**0.93**	Ms03_29h10	**0.44**	**2.92**
Ms03_20g12	**0.87**	**1.40**	Ms03_29h11	**0.51**	**0.93**
Ms03_20h01	**0.10**	**0.50**	Ms03_29h12	**0.09**	**2.02**
Ms03_20h02	**0.29**	**2.50**	Ms03_30a01	**34.10**	**0.57**
Ms03_20h03	**0.29**	**0.82**	Ms03_30a02	**4.24**	**0.79**
Ms03_20h04	**0.19**	**0.56**	Ms03_30a03	**0.36**	**2.46**
Ms03_20h05	**0.14**	**0.41**	Ms03_30a04	**0.40**	**0.74**
Ms03_20h06	**1.23**	**0.58**	Ms03_30a05	**0.56**	**1.43**
Ms03_20h07	**0.30**	**0.91**	Ms03_30a06	**2.12**	**1.07**
Ms03_20h08	**0.28**	**0.60**	Ms03_30a07	**0.98**	**9.11**
Ms03_20h09	**0.73**	**0.46**	Ms03_30a08	**1.04**	**4.83**
Ms03_20h10	**0.40**	**0.48**	Ms03_30a09	**1.06**	**1.15**
Ms03_20h11	**0.58**	**0.71**	Ms03_30a10	**1.40**	**2.80**
Ms03_20h12	**1.40**	**2.11**	Ms03_30a11	**1.13**	**2.02**
Ms03_21a01	**0.44**	**0.79**	Ms03_30a12	**4.26**	**1.11**
Ms03_21a02	**2.85**	**0.57**	Ms03_30b01	**0.88**	**0.21**
Ms03_21a03	**0.79**	**0.93**	Ms03_30b02	**0.41**	**1.15**
Ms03_21a04	**1.63**	**0.35**	Ms03_30b03	**0.11**	**1.99**
Ms03_21a05	**0.99**	**0.90**	Ms03_30b04	**0.60**	**1.90**
Ms03_21a06	**0.65**	**1.42**	Ms03_30b05	**0.34**	**1.04**
Ms03_21a07	**0.94**	**2.52**	Ms03_30b06	**0.94**	**0.63**
Ms03_21a08	**0.72**	**1.12**	Ms03_30b07	**0.21**	**1.86**
Ms03_21a09	**1.18**	**1.28**	Ms03_30b08	**0.44**	**7.50**
Ms03_21a10	**4.57**	**0.32**	Ms03_30b09	**1.78**	**1.54**
Ms03_21a11	**3.14**	**0.72**	Ms03_30b10	**1.43**	**3.42**
Ms03_21a12	**0.39**	**0.70**	Ms03_30b11	**0.82**	**1.36**
Ms03_21b01	**0.77**	**7.45**	Ms03_30b12	**1.04**	**1.43**

Ms03_21b02	**0.88**	**1.26**	Ms03_30c01	**0.06**	**1.30**
Ms03_21b03	**0.78**	**1.33**	Ms03_30c02	**1.19**	**1.96**
Ms03_21b04	**1.03**	**0.55**	Ms03_30c03	**0.55**	**0.59**
Ms03_21b05	**0.16**	**6.02**	Ms03_30c04	**2.40**	**2.08**
Ms03_21b06	**1.52**	**1.67**	Ms03_30c05	**1.01**	**1.21**
Ms03_21b07	**0.29**	**0.87**	Ms03_30c06	**1.04**	**1.25**
Ms03_21b08	**0.57**	**0.41**	Ms03_30c07	**0.58**	**1.31**
Ms03_21b09	**0.91**	**2.25**	Ms03_30c08	**0.19**	**0.92**
Ms03_21b10	**0.63**	**1.29**	Ms03_30c09	**1.50**	**1.52**
Ms03_21b11	**0.69**	**7.68**	Ms03_30c10	**0.17**	**4.29**
Ms03_21b12	**0.50**	**0.69**	Ms03_30c11	**0.50**	**4.29**
Ms03_21c01	**0.72**	**0.75**	Ms03_30c12	**0.35**	**0.08**
Ms03_21c02	**0.78**	**1.50**	Ms03_30d01	**0.11**	**1.29**
Ms03_21c03	**0.30**	**0.70**	Ms03_30d02	**1.39**	**1.30**
Ms03_21c04	**0.48**	**6.02**	Ms03_30d03	**0.87**	**2.68**
Ms03_21c05	**0.76**	**1.33**	Ms03_30d04	**2.11**	**0.46**
Ms03_21c06	**0.45**	**0.28**	Ms03_30d05	**1.04**	**1.25**
Ms03_21c07	**0.50**	**3.65**	Ms03_30d06	**0.82**	**2.81**
Ms03_21c08	**1.97**	**1.18**	Ms03_30d07	**1.41**	**0.86**
Ms03_21c09	**0.40**	**0.70**	Ms03_30d08	**0.99**	**0.72**
Ms03_21c10	**0.30**	**0.80**	Ms03_30d09	**1.10**	**0.79**
Ms03_21c11	**0.52**	**1.19**	Ms03_30d10	**7.49**	**1.82**
Ms03_21c12	**0.82**	**1.09**	Ms03_30d11	**0.38**	**3.86**
Ms03_21d01	**0.63**	**0.69**	Ms03_30d12	**0.62**	**2.32**
Ms03_21d02	**0.24**	**0.45**	Ms03_30e01	**0.16**	**0.44**
Ms03_21d03	**0.50**	**2.91**	Ms03_30e02	**1.94**	**1.13**
Ms03_21d04	**0.86**	**1.20**	Ms03_30e03	**0.59**	**14.40**
Ms03_21d05	**1.79**	**1.08**	Ms03_30e04	**0.33**	**1.11**
Ms03_21d06	**0.42**	**0.53**	Ms03_30e05	**0.41**	**3.47**
Ms03_21d07	**0.86**	**1.52**	Ms03_30e06	**0.33**	**1.28**
Ms03_21d08	**0.29**	**0.32**	Ms03_30e07	**0.86**	**3.21**
Ms03_21d09	**0.31**	**2.05**	Ms03_30e08	**1.00**	**1.01**
Ms03_21d10	**0.08**	**0.40**	Ms03_30e09	**4.00**	**0.84**
Ms03_21d11	**0.77**	**0.78**	Ms03_30e10	**2.03**	**0.78**
Ms03_21d12	**0.69**	**0.69**	Ms03_30e11	**0.68**	**1.89**
Ms03_21e01	**5.22**	**5.42**	Ms03_30e12	**0.07**	**1.21**
Ms03_21e02	**3.82**	**3.44**	Ms03_30f01	**0.76**	**0.65**
Ms03_21e03	**0.23**	**0.11**	Ms03_30f02	**0.24**	**2.66**
Ms03_21e04	**0.41**	**0.58**	Ms03_30f03	**0.15**	**1.59**
Ms03_21e05	**0.34**	**0.85**	Ms03_30f04	**0.51**	**3.32**
Ms03_21e06	**1.01**	**1.00**	Ms03_30f05	**0.80**	**0.73**
Ms03_21e07	**0.37**	**1.05**	Ms03_30f06	**0.54**	**0.71**
Ms03_21e08	**0.76**	**1.67**	Ms03_30f07	**0.74**	**7.98**
Ms03_21e09	**0.59**	**1.01**	Ms03_30f08	**1.09**	**0.74**
Ms03_21e10	**1.67**	**1.20**	Ms03_30f09	**0.39**	**2.57**
Ms03_21e11	**0.23**	**0.50**	Ms03_30f10	**0.24**	**1.63**
Ms03_21e12	**0.35**	**0.45**	Ms03_30f11	**0.26**	**0.92**
Ms03_21f01	**0.75**	**1.19**	Ms03_30f12	**0.46**	**1.00**
Ms03_21f02	**1.68**	**1.66**	Ms03_30g01	**0.96**	**1.32**
Ms03_21f03	**0.66**	**0.28**	Ms03_30g02	**1.31**	**6.98**
Ms03_21f04	**0.47**	**0.43**	Ms03_30g03	**2.23**	**1.32**
Ms03_21f05	**0.48**	**1.03**	Ms03_30g04	**0.47**	**0.46**
Ms03_21f06	**0.36**	**1.08**	Ms03_30g05	**1.29**	**1.10**
Ms03_21f07	**0.73**	**1.23**	Ms03_30g06	**1.55**	**0.82**
Ms03_21f08	**0.92**	**1.89**	Ms03_30g07	**0.47**	**0.79**
Ms03_21f09	**0.47**	**0.37**	Ms03_30g08	**0.79**	**0.55**
Ms03_21f10	**1.70**	**0.51**	Ms03_30g09	**0.44**	**2.69**
Ms03_21f11	**0.31**	**0.71**	Ms03_30g10	**0.22**	**3.25**
Ms03_21f12	**0.79**	**0.28**	Ms03_30g11	**0.63**	**1.73**

Ms03_21g01	**0.34**	**0.76**
Ms03_21g02	**0.61**	**1.23**
Ms03_21g03	**0.35**	**1.31**
Ms03_21g04	**0.20**	**0.43**
Ms03_21g05	**0.77**	**1.17**
Ms03_21g06	**0.67**	**0.47**
Ms03_21g07	**0.59**	**0.62**
Ms03_21g08	**0.24**	**0.65**
Ms03_21g09	**0.22**	**0.90**
Ms03_21g10	**0.27**	**0.27**
Ms03_21g11	**0.24**	**1.04**
Ms03_21g12	**0.59**	**0.78**
Ms03_21h01	**1.19**	**1.64**
Ms03_21h02	**0.94**	**0.52**
Ms03_21h03	**1.90**	**0.44**
Ms03_21h04	**0.76**	**0.41**
Ms03_21h05	**0.63**	**0.64**
Ms03_21h06	**0.34**	**1.68**
Ms03_21h07	**0.21**	**0.34**
Ms03_21h08	**0.72**	**0.83**
Ms03_21h09	**1.52**	**0.38**
Ms03_21h10	**0.52**	**0.81**
Ms03_21h11	**0.27**	**1.37**
Ms03_21h12	**0.34**	**5.08**
Ms03_22a01	**7.15**	**4.71**
Ms03_22a02	**0.65**	**2.16**
Ms03_22a03	**1.32**	**1.00**
Ms03_22a04	**0.88**	**2.20**
Ms03_22a05	**2.37**	**0.39**
Ms03_22a06	**1.54**	**0.68**
Ms03_22a07	**0.46**	**1.27**
Ms03_22a08	**0.25**	**0.56**
Ms03_22a09	**0.56**	**0.98**
Ms03_22a10	**0.36**	**0.49**
Ms03_22a11	**1.96**	**3.52**
Ms03_22a12	**0.42**	**0.50**
Ms03_22b01	**0.26**	**0.72**
Ms03_22b02	**0.17**	**0.16**
Ms03_22b03	**0.96**	**1.85**
Ms03_22b04	**1.62**	**0.53**
Ms03_22b05	**1.87**	**14.53**
Ms03_22b06	**0.59**	**0.84**
Ms03_22b07	**1.37**	**1.72**
Ms03_22b08	**1.81**	**3.76**
Ms03_22b09	**2.01**	**1.96**
Ms03_22b10	**0.19**	**5.58**
Ms03_22b11	**0.24**	**0.74**
Ms03_22b12	**0.54**	**0.54**
Ms03_22c01	**0.53**	**0.44**
Ms03_22c02	**0.57**	**1.51**
Ms03_22c03	**0.99**	**1.44**
Ms03_22c04	**1.08**	**0.86**
Ms03_22c05	**0.25**	**0.84**
Ms03_22c06	**0.46**	**15.69**
Ms03_22c07	**0.58**	**0.95**
Ms03_22c08	**1.47**	**5.31**
Ms03_22c09	**0.18**	**0.34**
Ms03_22c10	**0.22**	**0.55**
Ms03_22c11	**0.63**	**0.67**

Ms03_30g12	**0.39**	**0.57**
Ms03_30h01	**0.20**	**0.84**
Ms03_30h02	**1.18**	**1.41**
Ms03_30h03	**0.72**	**0.52**
Ms03_30h04	**0.33**	**1.19**
Ms03_30h05	**0.99**	**0.75**
Ms03_30h06	**5.24**	**0.49**
Ms03_30h07	**1.64**	**0.52**
Ms03_30h08	**0.78**	**0.45**
Ms03_30h09	**4.51**	**0.74**
Ms03_30h10	**1.88**	**0.89**
Ms03_30h11	**0.32**	**0.63**
Ms03_30h12	**0.20**	**0.99**
Ms03_33e12	**4.18**	**1.57**
Ms03_33f06	**0.59**	**1.29**
Ms03_33g12	**1.11**	**0.89**
Ms03_37g01	**0.65**	**0.89**
Ms03_38b12	**0.69**	**1.26**
Ms03_38c02	**0.72**	**2.90**
Ms03_39c02	**0.69**	**0.45**
Ms03_44f12	**1.24**	**0.87**
Ms03_45c01	**2.73**	**0.32**
Ms03_49a12	**0.46**	**0.73**
Ms03_49g10	**0.68**	**0.40**
Ms03_52a02	**0.33**	**0.31**
Ms03_58a10	**1.04**	**0.94**
Ms03_60a12	**0.85**	**1.10**
Ms03_60c02	**1.51**	**0.61**
Ms03_64c06	**0.81**	**1.84**
Ms03_66c02	**1.76**	**1.46**
Ms03_71e09	**0.54**	**0.72**
Ms03_75b07	**1.03**	**1.21**
Ms03_75b11	**0.80**	**1.44**
Ms03_77h02	**1.00**	**1.07**
Ms03_78f01	**0.20**	**2.23**

Ms03_22c12	**0.52**	**0.26**
Ms03_22d01	**1.55**	**1.28**
Ms03_22d02	**1.62**	**1.10**
Ms03_22d03	**1.54**	**0.80**
Ms03_22d04	**0.30**	**0.35**
Ms03_22d05	**0.97**	**1.39**
Ms03_22d06	**0.78**	**3.11**
Ms03_22d07	**0.68**	**0.68**
Ms03_22d08	**1.19**	**0.65**
Ms03_22d09	**0.63**	**0.84**
Ms03_22d10	**0.50**	**0.73**
Ms03_22d11	**0.61**	**0.96**
Ms03_22d12	**0.72**	**1.14**
Ms03_22e01	**0.26**	**1.12**
Ms03_22e02	**0.46**	**0.85**
Ms03_22e03	**0.34**	**0.80**
Ms03_22e04	**0.44**	**0.55**
Ms03_22e05	**0.31**	**0.57**
Ms03_22e06	**0.78**	**1.12**
Ms03_22e07	**0.79**	**1.26**
Ms03_22e08	**5.22**	**2.64**
Ms03_22e09	**0.20**	**0.34**
Ms03_22e10	**0.55**	**2.43**
Ms03_22e11	**0.15**	**0.46**
Ms03_22e12	**0.46**	**0.69**
Ms03_22f01	**0.36**	**0.81**
Ms03_22f02	**5.83**	**0.60**
Ms03_22f03	**1.16**	**0.87**
Ms03_22f04	**0.32**	**2.03**
Ms03_22f05	**1.12**	**0.27**
Ms03_22f06	**2.14**	**1.63**
Ms03_22f07	**1.28**	**0.58**
Ms03_22f08	**0.92**	**2.62**
Ms03_22f09	**0.53**	**0.65**
Ms03_22f10	**2.36**	**1.02**
Ms03_22f11	**0.67**	**1.32**
Ms03_22f12	**0.38**	**0.36**
Ms03_22g01	**0.23**	**1.26**
Ms03_22g02	**0.37**	**0.94**
Ms03_22g03	**0.51**	**1.13**
Ms03_22g04	**0.61**	**0.52**
Ms03_22g05	**0.98**	**1.36**
Ms03_22g06	**1.09**	**1.21**
Ms03_22g07	**0.62**	**1.74**
Ms03_22g08	**0.38**	**0.48**
Ms03_22g09	**0.55**	**0.83**
Ms03_22g10	**0.77**	**0.35**
Ms03_22g11	**0.78**	**0.95**
Ms03_22g12	**0.31**	**1.87**
Ms03_22h01	**0.71**	**0.67**
Ms03_22h02	**1.83**	**0.18**
Ms03_22h03	**0.48**	**0.57**
Ms03_22h04	**0.21**	**0.89**
Ms03_22h05	**0.73**	**0.86**