MICROTUBULE INVOLVEMENT IN THE PLANT LOW TEMPERATURE RESPONSE

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Kerry Sproule

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ABSTRACT

Cold acclimation is a complex process where plants acquire increased freezing tolerance following exposure to low, non-freezing temperatures. Microtubules are dynamic components of the cytoskeleton that are essential for plant growth and development, and there are multiple lines of evidence indicating microtubules are involved in the acquisition of freezing tolerance.

The organization of microtubules (MTs) was tracked over the course of a cold acclimation period using GFP:TUB6 and fluorescent imaging tools. Experiments found that MTs undergo incomplete, transient disassembly following exposure to acclimating temperatures, which is accompanied by intranuclear tubulin accumulation and followed by MT reassembly. The importance of the observed changes to MT organization was examined with MT disrupting chemicals that caused reduced MT dynamics or induced transient MT disassembly similar to that of cold acclimation. Results of these experiments suggest that MT reorganization is important for cold acclimation, but the disassembly and reassembly do not directly control cold acclimation.

MT binding proteins are likely to play a key role in the low temperature response because they control MT activity and organization, participate in low temperature signal transduction pathways, and mediate interactions between various elements of this pathway. By employing a number of proteomics techniques we were able to identify 96 tubulin-binding proteins from untreated and short term cold acclimated Arabidopsis plants. Proteins both known to and predicted to bind to MTs and unexpected MT binding proteins were identified. The identified tubulin binding proteins have a range of cellular functions, including RNA transport and protein translation, stress responses, and functions related to various metabolic pathways, and cell growth and organization.

Exposure to low temperatures affected the binding of some of these proteins to MTs with the identified tubulin binding proteins potentially involved in the cold acclimation process and stress response through a number of possible pathways.

This study represents the first live cell imaging of MT reorganization in response to low temperatures and the first time microtubule binding proteins from whole plant protein extracts were identified using 1D gel LC-MS/MS analysis.

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LIST OF ABBREVIATIONS

+TIPs	microtubule plus-end-tracking proteins
1D	one dimension
2D	two dimension
ABA	abscisic acid
AF	actin microfilament
AGP	arabinogalactan proteins
AIR9	auxin-induced root culture 9
BSA	bovine serum albumin
C	control
CBF	C-repeat binding factor
CLASPs	CLIP170-associated proteins
COR	cold-responsive
d	day(s)
DIGE	differential in-gel electrophoresis
DMSO	dimethyl sulfoxide
DRP	dynamin related protein
DRE/CRT	dehydration-responsive element/C-repeat
DREB	dehydration-responsive element-binding protein
EB1	end binding protein 1
EC	electrical conductivity
eEF1α	translation elongation factor 1α
eFP	electronic Fluorescent Pictograph Browser
ER	endoplasmic reticulum
ERD	early dehydration inducible
ESK1	Eskimo1
EST	expressed sequence tag
FAD	flavin adenine dinucleotide
FP	fluorescent protein
FRY1	Fiery 1
GA	gibberellic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAP	GPI anchored protein
GAST1	gibberellic acid stimulated transcript 1
GDP	guanosine diphosphate

GFP	green fluorescent protein
GhKCH2	*Gossypium hirsutum* kinesin with calponin homology
GPI	glycosyl phosphatidylinositol
GRP	glycine-rich protein
GTP	guanosine triphosphate
h	hour(s)
HK1	histidine kinase 1
HOS	high expression of osmotically responsive genes
ICAT	isotope-coded affinity tagging
ICE	inducer of CBF expression
InsP	inositol polyphosphates
iTRAQ	isobaric tags for relative and absolute quantification
kDa	kilodalton
KIN	cold inducible
KO	gene knockout
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LRR	leucine-rich repeat
LT	low temperature treated
LT_{50}	lethal temperature killing 50% of population
LTI	low temperature inducible
LTP	lipid transfer protein
MAPK	mitogen-activated protein kinase
MAP	microtubule associated protein
MCO	multi-copper oxidase
MF	microfibril
MFP	multifunctional protein
MOR1	microtubule organization 1
mRNA	messenger RNA
MS	Murashige and Skoog medium
MSDB	Mass Spectrometry protein sequence DataBase
MS/MS	tandem mass spectrometry
MT	microtubule
MW	molecular weight
m/z	mass to charge ratio
NA	numerical aperture
NPC	nuclear pore complex

OD	optical density
PAGE	polyacrylamide gel electrophoresis
PERK	proline-rich extensin-like receptor kinase
PH	pleckstrin homology
Phi	phosphate-induced
PLC	phospholipase C
PLD	phospholipase D
PM	plasma membrane
PPB	prephophase band
PTM	post-translational
ROS	reactive oxygen species
SAMS	Southern Alberta Mass Spectrometry
SB401	*Solanum berthaultii* pollen-specific protein
SDS	sodium dodecyl sulfate
SFR	sensitive to freezing
Spc98p	Spindle pole body component of 98 kDa
smRS-GFP	red-shifted version of GFP
SOS	Salt overly sensitive
SPR	Spiral
TAN1	Tangled 1
TUA	alpha-tubulin
TUB	beta-tubulin
WAK	Wall-associated kinase
wk	week(s)
wt	wild type
WVD2	Wave-dampened 2

CHAPTER 1
LITERATURE REVIEW

1.1 The Plant Cytoskeleton

The cytoskeleton is a key structure contributing to the organization of all eukaryotic cells. Microtubules (MTs) are the largest component of the plant cytoskeleton network, and are critical elements for all cellular functioning, from cell morphogenesis, cell division, cell wall formation, to the intercellular transportation of vesicles and nucleotides (mRNA and DNA-chromosomes) (Fosket and Morejohn 1992; Moore et al. 1997; Nick 2000). The term cytoskeleton is misleading, as it implies that the elements are rigid and only function to maintain structure, when they are in fact very dynamic elements that continually reorganize into different forms and are involved in complex activity, interactions, and regulation (Wasteneys and Galway 2003). Although MTs are comprised primarily of groups of the same small protein subunits, these subunits are encoded by many genes, which create proteins with unique features that can be modified in various ways. An additional diverse array of proteins associate with MTs and function to not only control the activity and organization of the MTs but also facilitate interactions with other cellular components and potentially regulate signalling pathways.

1.1.1 Microtubule Organization and Dynamics

1.1.1.1 Plant microtubule structure and arrays

The major component of MTs is the dimeric protein tubulin. Tubulin most often exists in the form of non-covalently linked 50,000 dalton α- and β-tubulin polypeptides (Fosket and Morejohn 1992). The structure of a MT and tubulin dimer is shown in Figure 1.1. Alpha- and β-tubulin dimers bind end to end to form linear protofilaments, which interact laterally to form the wall of a hollow MT (Figure 1.1C). Typically, thirteen protofilaments join to form a hollow, 25 nm diameter MT (Figure 1.1). Contact between protofilaments is mediated by interactions between the N and M loops of laterally adjacent subunits (Morrissette et al. 2004). In addition to α- and β-tubulin, MTs contain a relatively small amount of the protein γ-tubulin, which acts in conjunction with guanosine triphosphate (GTP) to facilitate MT nucleation (Cyr 1994; Drykova et al. 2003).

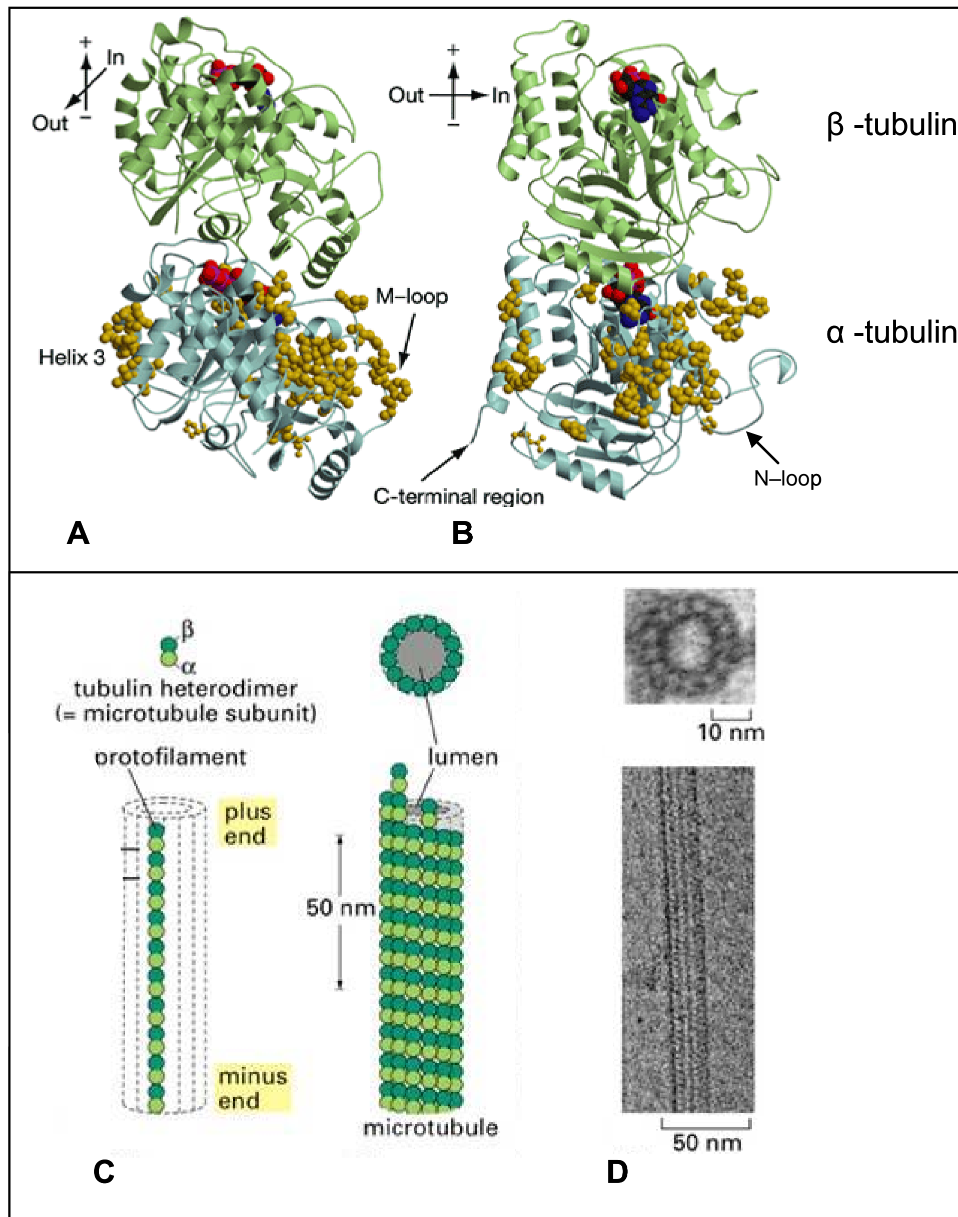

Figure 1.1. MT and tubulin structure. (A and B) Tubulin heterodimer comprised of tightly linked α- and β-tubulin monomers. (A) View from the outside of the MT. (B) View from the M-loop side of a protofilament where protofilaments interact with each other laterally. GTP molecules within the proteins are shown in red and dark blue. The yellow circles highlight amino-acid side chains which differ between α-tubulin isoforms in yeast. Most of the variation occurs at the exterior protein surface. (C) Diagram of a tubulin dimer, single protofilament and MT. Each protofilament consists of adjacent tubulin subunits with the same orientation, and a MT consists of 13 protofilaments aligned in parallel around the lumen. (D) A segment of a MT viewed in an electron microscope with a cross section of a MT showing a ring of 13 protofilaments. Images A - B courtesy Bode et al. (2003); images C - D courtesy Molecular Biology of the Cell, 4th Edition.

Plants are distinguished by the presence of a cell wall and chloroplasts, and accompanying the evolution of these unique structures was the evolution of unique cytoskeletal arrays. Plant MT arrays include the preprophase band and phragmoplast in addition to the flexible cortical array and spindle array that are present in all eukaryotes (Wasteneys 2002). The dispersed cortical MTs are important for regulating the direction of cell expansion, controlling cell wall growth, directing Golgi vesicles to the developing wall, and aligning new cellulose microfibrils (MFs) on the wall, among other functions (Wasteneys and Yang 2004). The other three MT arrays are primarily responsible for different aspects of cell division, including the separation of sister chromatids and proper formation and alignment of the cell plate (Cowling et al. 2003).

1.1.1.2 Microtubule dynamics

MTs are very dynamic polymers that continually reorganize and undergo cycles of growing, pausing, and shortening. Individual MTs alternate between growing and shrinking by the rapid attachment and detachment of the subunits at their ends (Stoppin-Mellet et al. 2003). Treadmilling and dynamic instability are two forms of MT dynamics characteristic of plant MTs. Treadmilling occurs when tubulin dimers add preferentially to one end of the MT, while being slowly lost from the other end. Treadmilling is a form of directional growth; the rapidly growing end is considered the plus end and the slow growing end is called the minus end (Bisgrove et al. 2004). The term 'dynamic instability' describes the variable nature of MT plus end dynamics, where some of the MTs in the population undergo continual slow growth, while others disassemble very rapidly (Sedbrook 2004). When MTs 'move,' the individual tubulin dimers of the MT do not slide or move in relation to the cell. Instead, movement is caused by the dynamic loss and addition of tubulin dimers at the MT ends (Shaw et al. 2003; Van Damme et al. 2004).

Plant MT dynamics are different from animal MT dynamics. Plant MTs are almost always moving, the relatively 'static' interphase MTs are only in a pause state about 10% of the time (Shaw et al. 2003). Individual plant MT plus ends polymerize at an average rate of 3–5 μm/min and depolymerise at approximately 5–9 μm/min, which is three times slower than animal MTs (Vos et al. 2004). Animal MT dynamics are reduced overall because MT minus ends generally remain stably attached to their nucleation sites (see below), resulting in reduced overall dynamicity and states of pause that occupy 60% of the MT lifetime (Ehrhardt and Shaw 2006).

1.1.1.3 Tubulin dimer assembly and microtubule nucleation

The cell tightly regulates all aspects of tubulin production, from the production of tubulin mRNA to protein translation and dimer formation. The α- and β-tubulin polypeptides are modified by a sequence of chaperonins and tubulin folding cofactors before they are assembled into dimers (Dhonukshe et al. 2006). This step in MT assembly is one of many important mechanisms of MT regulation that controls both the ratio of α- to β-tubulin monomers and the ratio of free tubulin dimers to MTs (Dhonukshe et al. 2006). The proper balance between each element is maintained in part by complex feedback mechanisms that control the production and degradation of tubulin transcripts and polymers (Giani et al. 2002). High ratios of free tubulin dimers to MTs generally result in increased rates of MT polymerization, but if an increased polymerization does not significantly reduce free tubulin levels, mechanisms of protein and/or transcript degradation will be initiated. Imbalances between α- and β-tubulin are known to decrease MT numbers, and the introduction of artificially high levels of either α- or β-tubulin without the counterpart has been shown to be toxic in some cells because of their inability to properly correct the imbalance (Weinstein and Solomon 1990; Anthony et al. 1998; Dhonukshe et al. 2006).

MT nucleation, the initial formation of a MT, occurs when the ring shaped γ-tubulin ring complex, consisting of γ-tubulin and a few other elements, forms around one or two tubulin dimers (Erhardt et al. 2002). This structure is called a MT initial, and its presence encourages the binding of more dimers (Hashimoto and Kato 2006). The growth of this initial into a MT involves the addition of GTP-bound tubulin dimers onto the MT plus end, producing a GTP cap (Vassileva et al. 2005). The presence of the GTP cap promotes the addition of more GTP bound tubulin dimers. Shortly after the polymerization of the new tubulin dimers, the GTP is hydrolyzed by β-tubulin, inducing a conformational change in the MT that favours MT shortening by the loss of tubulin dimers (Bisgrove et al. 2004). The MT minus end can be capped and stabilized by a group of nucleating proteins that prevent the addition of new tubulin dimers while still permitting depolymerization (Shaw et al. 2003).

MT nucleation does not occur at a defined nucleating site in plant cells, unlike the specific microtubule organizing center (MTOC) of yeast and animal cells. Instead, new MTs originate off of the loose MTOC or the nuclear envelope and as branches off of pre-existing MTs. The MT-dependent nucleation that forms cortical MTs occurs when γ-tubulin is recruited

to the pre-existing MTs (Murata and Hasebe 2007). From the site of nucleation, MTs can grow in any direction within the two-dimensional plane around the cell cortex. In land plants new cortical microtubules usually form at an approximate 40° angle to the original microtubule (Murata and Hasebe 2007). MTs can grow in slightly curved or straight lines, and when a growing MT intersects a pre-existing MT it either crosses over the MT (if intersecting at angles above 30–40°) or changes its direction and becomes aligned and often bundled with the contacted MT (if intersecting at angles below 30–40°) (Ehrhardt and Shaw 2006). Because of minus end dynamics, the part of the MT not forming into the bundle is less stable than the bundled MTs and usually depolymerises, leaving only the parallel, bundle-stabilized MTs (Shaw et al. 2003). These characteristics are responsible for the self-organization of cortical MTs into the relatively parallel, organized MT arrays commonly found in plant cells (Ehrhardt and Shaw 2006).

1.1.2 Tubulin Genetics and Microtubule Heterogeneity

1.1.2.1 Tubulin genes and post translational modifications

In plants, the α- and β-tubulins are encoded by two small gene families. These gene families are well characterized in the small model plant *Arabidopsis thaliana* (hereafter referred to as Arabidopsis), with six expressed α-tubulin genes (Kopczak et al. 1992), nine expressed β-tubulin genes (Snustad et al. 1992), and two expressed γ-tubulin genes (Drykova et al. 2003). Excluding a highly variable carboxyl-terminal domain, all α- and β-tubulin isoforms exhibit a high degree of sequence identity. Plant tubulin amino acid sequences share at least 87% identity amongst the β-tubulins, and at least 89% identity between the α-tubulins (Fosket and Morejohn 1992). Plant tubulins are also highly conserved with tubulins from other organisms; angiosperm and vertebrate tubulins share at least 79% identity (Fosket and Morejohn 1992). Tubulin proteins from all organisms maintain the same basic and specific activity within the MT, including MT assembly and disassembly, α- and β-tubulin interaction, and GTP hydrolysis. The high degree of tubulin isotype similarity across all organisms relates to this highly conserved tubulin activity. Protein sequence variation that is found in different tubulin proteins is largely localized to residues present at the exterior protein surface (Figure 1.1A and B).

There are more tubulin genes present in plants than any other organism, with 17 expressed tubulin genes in the small (1.3×10^8 bp) genome of Arabidopsis compared to only 15 expressed tubulin genes in the human genome (3×10^9 bp) (Goddard et al. 1994). In addition to the large number of tubulin isoforms, tubulin heterogeneity is also produced by posttranslational

modifications (PTMs). In plant systems detyrosinated/tyrosinated, acetylated/deacetylated, polyglutamylated and phosphorylated tubulin have been found (Koontz and Choi 1993; Smertenko 1997; Wang et al. 2004).

1.1.2.2 Possible functional effects of tubulin heterogeneity

It is believed that the large number of plant tubulin genes are present to help the plant better adapt to their environment, but the actual functional significance of the apparent gene redundancy is not well understood (Goddard et al. 1994). Three explanations have been proposed; i) different tubulin isoforms are required to perform particular functions, ii) different tubulin isoforms are functionally interchangeable, and the presence or prominence of a specific isoform is caused by preferential expression driven by different promoters, and iii) tubulin isoforms coevolved along with microtubule-associated proteins that interact with microtubules, and specific isoforms are selected based on their interaction with a particular set of these proteins (Goddard et al. 1994; Breviario and Nick 2000). Tubulin composition varies both spatially and temporally by both differential transcription of tubulin genes and regulated modifications of α- and β-tubulin by PTMs. Some tubulin genes are constitutively expressed while others have specific patterns of expression and regulation, based on both developmental stage and environmental conditions (Goddard et al. 1994; Cheng et al. 2001; Abdrakhamanova et al. 2003). For example, in Arabidopsis, the *TubA2* gene is expressed in nearly all tissues at all times, whereas *TubA1*, *TubB1*, and *TubB9* are preferentially expressed in root cortical cells, leaf tissues, and mature pollen and stamens, respectively (Nick 2000; Cheng et al. 2001). External factors including light and temperature also affect tubulin isotype expression. In Arabidopsis, light decreases the levels of *TubB1*, while low temperature decreases transcript levels of *TubB2, TubB3, TubB6,* and *TubB8*, and increases levels of *TubB9* (Chu et al. 1993). Low temperature causes complex cyclic patterns of tubulin gene expression in wheat (Farajalla and Gulick 2007).

Modified plant tubulins have been observed, but relatively little information is available on tubulin PTMs. Plant tubulin PTMs seem to occur in a highly specific manner, only to particular tubulin isoforms and in specific tissue types (Wang et al. 2004). Acetylated α-tubulin in MT arrays is associated with stable MT structures in animal systems and may mark less dynamic MTs in plant systems as well, while the presence of tyrosinated tubulin in MTs usually indicates that the MT is quite new (Åström 1992; Smertenko 1997). Phosphorylation of tubulin

has been associated with cell cycle progression and programmed cell death in plants (Yemets et al. 2005).

Although the functional importance of post-translational modifications is not obvious, one proposed function is the creation of tubulin/MT subpopulations that are 'targeted' for specific functions (Wang et al. 2004). At least some of the modifications (e.g. acetylation and polyglutamylation) act to regulate the binding of MAPs to MTs (Westermann and Weber 2003). Hormone activity also affects PTMs, and some PTMs can be triggered by signals that control plant growth (Duckett and Lloyd 1994; Wiesler et al. 2002).

Differential expression and modification of tubulin genes and proteins under different conditions suggests that stress may be better managed/tolerated because of these protein alterations. For example, MT stability during chilling appears to be determined by features of β-tubulin C-termini. Antarctic fishes contain MTs that are extremely tolerant to low temperatures and can polymerize at very low temperatures (Detrich and Parker 1993). Low temperature tolerant fish contain β-tubulin proteins with short C-termini that are less glutamylated than other β-tubulin isoforms (Redeker et al. 2004). These MTs are typically much less dynamic than homeotherm MTs at all temperatures (Detrich et al. 2000). Maize microtubules displayed increased cold tolerance and more closely resembled low temperature stable fish β-tubulins when the β-tubulin C-terminus was shortened (Bokros et al. 1996). In Arabidopsis, transcript levels of *Tub9* are increased and *Tub2*, *Tub3*, *Tub6* are decreased after approximately 6 hour (h) exposure to 4°C (Chu et al. 1993). TUB9 is the Arabidopsis tubulin protein with the shortest C-terminus (which by inference is most likely to be low temperature stable) while TUB2, TUB3 and TUB6 are larger proteins with longer C-termini.

Different features of the tubulin C-terminus may increase MT cold stability. The additional length of the protein ends may itself make MTs more prone to disassembly. The increased cold tolerance of the shorter tubulin isoforms could also be related to the decreased PTMs, as all PTMs except acetylation occur in the highly variable C-terminal protein end. Tubulin heterogeneity in plant MTs also appears to increase the rate of microtubule shortening, as MTs comprised of only one tubulin isoform (tubulin homopolymers) have much slower depolymerization rates than tubulin heteropolymers, which may have an evolutionary and functional significance (Moore et al. 1997).

1.1.3 Microtubule Associated Proteins (MAPs)

Microtubule associated proteins (MAPs) are important components of MTs. They regulate nearly all aspects of tubulin biochemistry and control everything from polymer stability, formation and elongation, to MT bundling and linkage to other cellular structures (Maiato et al. 2004). Traditionally MAPs referred only to proteins that bound directly to MTs, and a lot of recent debate and controversy has surrounded the defining of a MAP (Morejohn 1994; Sedbrook 2004). In this and other reports the definition of MAPs is considered to include proteins that indirectly or transiently interact with MTs, colocalize with MTs, or influence MT growth dynamics in some way (Hamada 2007).

A variety of MAPs have been identified in plants and a number of these have been characterized. Some characterized plant MAPs have structures and functions not found in other eukaryotic MAPs, while many of the major animal cytoskeletal proteins are absent from plants (Hussey et al. 2002; Wasteneys and Galway 2003). The plant cytoskeleton is quite unique, and the presence of the cell wall and lytic vacuoles, in combination with the lower levels of MTs in plant tissues have made the study of plant MTs challenging. Plant MAPs have been identified through a variety of approaches; including mutant screens, biochemical purifications, and identification of plant homologues to the much better characterized animal MAPs via database searching and antibody cross reactivity (Whittington et al. 2001; Chuong et al. 2004; Hamada 2007). Recently a large number of tubulin binding proteins were identified by a large-scale study employing tubulin affinity chromatography and mass spectrometry proteomic techniques (Chuong et al. 2004).

In general, plant MAPs can be grouped into two major functional categories, those that regulate MT assembly/disassembly, and those that organize MT structure and function (Hamada 2007). However, proteins associate with MTs that do not necessarily fit into these categories. The cytoskeleton filaments form a large, near continuous connection between nearly all cellular structures and provide a large surface area on which proteins can bind, and possibly carry out functions unrelated to cytoskeleton activity (Janmey 1998).

1.1.3.1 Microtubule disassembling and assembling MAPs

There are two different ways plant MTs are depolymerised: by low temperature induced depolymerization and by MT-severing proteins (Sedbrook 2004). Katanin is the only depolymerizing MAP that has been reported in plants, and it severs MTs through an ATP-

dependent mechanism (Burk et al. 2001). In animal systems, katanin functions as a heterodimer, with the p80 subunit bringing the heterodimer to the MT and regulating the p60 that severs the MT. Homologues of genes for both subunits exist in Arabidopsis but it seems that the p60 subunit acts alone; no p80 activity has been described to date (Stoppin-Mellet et al. 2003).

In plants, katanin is required for normal cell wall formation, cell elongation, cell expansion, and progression through the cell cycle where it breaks down MT arrays after their role in the cell cycle is fulfilled (Burk et al. 2001). MT depolymerizing MAPs are abundant in animals where they are responsible for detaching MT minus ends from stable MT nucleation sites, which is important for MT organization and function (Hamada 2007). It is possible that plants have more depolymerizing MAPs than just katanin, however, MT minus ends in plants are inherently more dynamic than in animals, making depolymerizing proteins much less important for plant MT array dynamics and organization.

MT nucleation requires γ-tubulin and a complex of other proteins, and because plant MTs are primarily nucleated on the surface of pre-existing MTs, the proteins in this complex are therefore also classified as MAPs. Unfortunately, the identity of proteins in this complex is not known (Pastuglia and Bouchez 2007). Spc98p is a MAP essential for MT nucleation in animals and its homologue has been examined in plants. However, although in plants Spc98p colocalizes with γ-tubulin at MT nucleation sites at the surface of the nucleus where it functions in MT nucleation, it does not localize along cortical MTs and is therefore unlikely to be involved in MT-dependent MT nucleation of cortical MTs (Erhardt et al. 2002).

MAP215 is a large, highly conserved MAP family present in all examined eukaryotes that control MT length by promoting both the attachment and detachment of tubulin dimers into MTs (Kawamura et al. 2006; Hamada 2007). MOR1 (microtubule organization 1) is an Arabidopsis member of the MAP215 family. MOR1 localizes in small patches along the entire length of MTs, resembling, and possibly correlating to MT nucleation sites (Hamada 2007). MOR1 stabilizes the growing ends of newly forming MTs where it is controls MT length throughout the cell cycle (Kawamura et al. 2006). MT organizational defects in *mor1* mutants caused aberrant cell plates, defective chromosomal arrangements and multinucleate cells revealing the importance of MOR1 and regulated MT length for proper MT array organization and function (Kawamura et al. 2006).

1.1.3.2 Microtubule organizing MAPs

1.1.3.2.1 Microtubule binding motor proteins. There are two groups of motor proteins that act on MTs, dyneins and kinesins. Dyneins organize the Golgi apparatus, establish spindle poles, and move nuclei, vesicles, and chromosomes in those organisms where they are found (Lawrence et al. 2001). However, only a single light chain dynein sequence has been identified in plants, and this protein does not appear to have any motor protein function (Reddy and Day 2001).

Kinesins are a superfamily of MT binding motor proteins that participate in diverse functions, generally related to organelle transport and the organization of MT structures. All eukaryotic organisms have kinesin proteins, which share a conserved motor domain and use energy from ATP hydrolysis to walk along MTs (Reddy and Day 2001). These motor proteins can translocate to both the plus and minus end of the MT, and can transport cargo to their respective target sites. Flowering plants have the most kinesin genes of any organism, and with 61 kinesins and kinesin-like genes Arabidopsis contains the most kinesins of all eukaryotic genomes sequenced (Reddy and Day 2001; Dagenbach and Endow 2004). There are 14 groups of kinesins grouped based on conserved motor domain structure (Dagenbach and Endow 2004). Two of these groups consist solely of plant kinesins, and many of the plant kinesins classified into the other families, particularly the kinesin 14 family, are quite divergent from the rest of their respective groups (Reddy and Day 2001; Dagenbach and Endow 2004).

The kinesin 14 C-terminal motor group is the most divergent of all the kinesin subfamilies and appears to function quite differently than other plant and animal kinesins (Reddy and Day 2001). Plants do not contain dynein motor proteins, and the divergent plant kinesin 14 proteins may perform functions carried out by these proteins in other organisms (Dagenbach and Endow 2004). Members of the kinesin 14 group are proposed to organize spindle MTs and can move MTs in relation to one another (Ambrose et al. 2005). A kinesin 14 protein from cotton, GhKCH2, appears to be involved in MT functioning during cell wall formation, by participating in microtubule dynamics and organization, or transporting materials during cell wall formation (Xu et al. 2007). Members of the kinesin 14 subfamily include kinesins with actin and calmodulin binding domains (Preuss et al. 2004).

TANGLED 1 (TAN1) is a MAP originally identified in maize that functions to control the position of cell division (Smith et al. 1996). TAN1 is present around the plane of cell division

throughout mitosis and cytokinesis, first with the preprophase band (PPB) and then alone at the cell periphery after the PPB disassembles, where it appears to continuously direct the expansion of the phragmoplast in the proper division plane (Walker et al. 2007). Two kinesins associate with TAN1, and mutations to any of these three proteins results in abnormal orientation of the cell plate, indicating that the function of these two kinesins is directly linked to the function of TAN1 (Smith et al. 2001; Hamada 2007).

1.1.3.2.2 MT plus-end-tracking proteins (+TIPs). MT plus-end-tracking proteins (+TIPs) are a diverse group of proteins that locate preferentially at MT plus ends, usually promoting the formation of longer and more active MTs (Bisgrove et al. 2004). The presence of these proteins generally increase MT dynamics thereby increasing the probability that a growing MT will interact with other MTs or proteins, becoming stabilized and retained for a specific function (Bisgrove et al. 2004). In this way +TIPs help bind MTs to localized cell sites, and orient them for proper polarized growth, spindle positioning, directional migration and plane of cell division (Bisgrove et al. 2004).

+TIPs are characterized by the formation of a "comet" that tracks the plus end of MTs when the +TIP is expressed as a green fluorescent protein (GFP) fusion protein. +TIPs continually hop on and off the MT plus end as it grows (Bisgrove et al. 2004). There are three distinct ways +TIPs are targeted to the MT ends; motor driven transport, treadmilling, and hitchhiking. Motor driven transport depends on kinesin motor proteins that essentially walk along MTs carrying +TIPs with them towards the plus end, where they unload their cargo. Treadmilling +TIP transport is based on unique chemical and/or structural properties of the tip, such as the GTP cap or copolymerization with tubulin dimers, which facilitates selective protein binding at the MT end. Hitchhiking +TIPs locate to the MT end by indirectly binding to MTs through a treadmilling protein intermediary.

End binding 1 (EB1), the first +TIP found in plants, is characteristic of direct binding, treadmilling +TIPs (Chan et al. 2003). Two EB1 proteins in Arabidopsis, AtEB1a and AtEB1b, regulate MT dynamics and promote the attachment of MT ends to different cellular sites. They localize at the MT plus end and can bind MT minus ends and nucleation sites where they appear to promote MT stabilization by helping anchor MTs to the cortex (Chan et al. 2003).

AtEB1b also binds to internal membranes, including the endoplasmic reticulum (ER), chloroplasts, mitochondria, and nuclei, facilitating organelle movement throughout the cell, as

well as being essential for organizing membranes and maintaining polar growth in higher plant cells (Mathur et al. 2003; Bisgrove et al. 2004). AtEB1 +TIPs can also act as an intermediary protein for other hitchhiking +TIPs. A number of EB1 associated proteins have been characterized in both plant (e.g. TAN1, MOR1 and the formins) and non-plant systems (Bisgrove et al. 2004).

CLASPs (CLIP170-associated proteins) are evolutionary conserved +TIPs with essential roles in mitosis and morphogenesis (Kirik et al. 2007). Although plants do not contain CLIP170 proteins, CLASP homologues have been identified in plants, and AtCLASP appears to function as a +TIP that localizes along cortical MTs, enriched at the MT plus end (Kirik et al. 2007). AtCLASP appears to be involved primarily in regulating cortical microtubule organization and cell form and expansion (Kirik et al. 2007).

SPIRAL 1 (SPR1) and SPIRAL 2 (SPR2) are plant specific MAPs involved in controlling the direction of cell elongation by interacting with cortical MTs (Ishida et al. 2007). Mutations in either SPR1 or SPR2 cause right handed helical growth in Arabidopsis seedlings and it is believed that both proteins act to control MT organization via separate pathways (Shoji et al. 2004). SPR1 is a very small (12 kD) +TIP protein that is localized at growing ends of MTs of the cortical array, spindle, phragmoplast and preprophase band through either direct binding to MTs or binding to other MAPs (Sedbrook et al. 2004). However, SPR2 is not a +TIP, and directly binds MT in discrete patterns along the length of the MTs (Shoji et al. 2004).

1.1.3.2.3 Microtubule bundling proteins: MAP65 and other candidates. In plants, the best-studied structural MAPs are cross bridging MAPs that belong to the MAP65 protein family. MAP65s are a broad grouping of proteins 54 to 80 kDa in size that appear to be unique to plants (Wasteneys 2002). Arabidopsis contains nine MAP65 genes, and the structure and function of many of these have been analyzed (Hussey et al. 2002). MAP65s have been shown to localize to cortical MTs during interphase, but MAP65 binding is cell cycle specific and different family members differentially localize to the preprophase band, phragmoplast and the midzone (Hamada 2007). MAP65 proteins form homodimers, and each protein of the dimer binds to the C-terminal region of tubulin in MTs to form a 10 – 30 nm crossbridge between two MTs (Smertenko et al. 2004). The binding of these proteins helps keep MTs parallel and properly spaced, and suppresses cold-induced MT disassembly (Smertenko et al. 2004; Wicker-Planquart et al. 2004). At least one MAP65 family member, AtMAP65-1, is regulated by phosphorylation,

as its phosphorylation is essential for its MT binding activity (Smertenko et al. 2004). *atmap65-1* mutants expand irregularly, produce a distorted phragmoplast and undergo unsuccessful cytokinesis (Muller et al. 2004). WAVE-DAMPENED 2 (WVD2), AUXIN-INDUCED ROOT CULTURE 9 (AIR9), and AtMAP70 are three recently characterized plant MAPs with limited to no identity to MAPs of other organisms (Kaloriti et al. 2007). WVD2 is a novel MAP in Arabidopsis that controls cortical MT bundling and array organization in elongating cells (Perrin et al. 2007). AIR9 binds to MTs in complex patterns, and appears to function in controlling the maturation of cell plates into cell walls (Kaloriti et al. 2007). Arabidopsis MAP70-1 binds to MTs in all MT arrays, but its function is unknown. It shares no similarity to other MAPs, and has no homologs in non-plant species (Kaloriti et al. 2007).

1.1.3.2.4 Actin-binding and membrane binding MAPs. Plasma membrane (PM) and MTs are closely linked, with cross bridges connecting cortical MTs to the PM having been observed by electron microscopy in protoplasts, roots, and other expanding tissue from a range of lower and higher plant species (Hardham and Gunning 1978; Dugas et al. 1989; Shibaoka 1994). Phospholipase D (PLD) is one PM protein that may be responsible for linking cortical MTs to the PM (Gardiner et al. 2001). The activation of PLD by n-butanol, osmotic stress and xylanase all have been shown to stimulate MT reorganization and the release of MTs from the PM (Gardiner et al. 2001; Dhonukshe et al. 2003). However, it is possible that phospholipase activators themselves promote MT depolymerization in the absence of PLD (Hamada 2007). Other PM - MT binding proteins have been found in tobacco (Cai et al. 2005), and a novel MAP recently identified from *Solanum berthaultii* pollen, SB401, may function to mediate interactions between MTs and membrane organelles (Huang et al. 2007).

The function and localization of MTs and actin microfilaments (AF) have often been found to overlap (Shibaoka 1994; Sonesson and Widell 1998; Wasteneys and Galway 2003). There are more than 10 types of proteins that cross-bridge MTs and AFs in animal and yeast cells. Such cross-bridging proteins have not yet been clearly identified in plants, only two proteins with the potential to crosslink these structures have been described. Tobacco MAP190 binds to both MTs and actin filaments in vitro, and the cotton kinesin, GhKCH2, colocalizes with both AFs and MTs in cotton fibres (Preuss et al. 2004; Hamada 2007). The SB401 protein also binds to and bundles AFs, and could connect them to MTs (Huang et al. 2007).

1.1.3.2.5 Other MAPs. It has been demonstrated that numerous proteins involved in a diverse array of cellular functions bind to MTs and it is unlikely that all of these proteins act as typical MAPs involved in controlling MT function/organization (Janmey 1998). Rather, some of these proteins may bind to MTs because of their association with other MAPS, or because the MT provides a relatively stable 'docking place' for the protein to carry out its function (e.g. machinery involved in translation) (Janmey 1998). Glycolytic enzymes, kinases, and GTPases are some examples that localize to MTs in animal and plant cells (Janmey 1998). MTs appear to be important for intracellular trafficking and can transport organelles (Muench and Mullen 2003; Romagnoli et al. 2003). One well-studied example of this is the peroxisomal multifunctional protein (MFP), which localizes along MTs to carry out its peroxisome function and help regulate mRNA localization and/or translation (Chuong et al. 2002; Muench and Mullen 2003).

Ribosomes, RNA and RNA-related proteins localize to the cytoskeleton, and mRNA transportation, translation and nuclear RNA export may be controlled in part by these interactions (possibly via MAPs) (Mandelkow and Mandelkow 1995; Jansen 1999). The translation elongation factor 1α (eEF1α) is a well studied MAP capable of binding and bundling actin filaments as well as its functions in protein synthesis and other activities (Durso and Cyr 1994; Moore and Cyr, 2000). Research in animals suggests that the binding of eEF1α to actin filaments inhibits the translational activity of eEF1α (Lopez-Valenzuela et al. 2003).

Very few large-scale analyses of proteins that bind to MTs and tubulin have been performed. Forgacs et al. (2004) performed an analysis of data from two independent large-scale yeast two-hybrid screens to identify and characterize the cytoskeleton proteome of *S. cerevisiae*. Actin, tubulin, proteins that bind actin or tubulin, and proteins that bind to a protein that binds actin or tubulin were all considered to be cytoskeleton proteins. Using this criterion, the authors identified 125 proteins that comprise the cytoskeleton proteome, representing 2.2% of the total yeast proteome.

A recent large scale analysis of plant tubulin binding proteins identified 122 proteins capable of binding tubulin, of which only 6% were previously known to either directly bind to or modify MTs, while just over 50% were related to proteins previously found to associate with MTs (Chuong et al. 2004). For many of these proteins, further analysis is required to confirm these interactions in vivo, and to determine if these interactions are important to the functioning of either MTs or the protein. The proteins identified in this study as MAPs confirms the

cytoskeleton as an important element in metabolic channelling, intracellular trafficking and signalling in plant cells.

1.1.4 Microtubule Disrupting Chemicals

The dynamic nature of MTs is susceptible to pharmacological agents, which are generally considered either MT-disrupting or MT-stabilizing chemicals. Taxol™ (paclitaxel) is a MT stabilizer that lowers the critical concentration of tubulin required for MT assembly and makes MTs more resistant to disassembly (Schiff et al. 1979; Nakamura et al. 2004). Taxol stabilizes MTs by binding to β-tubulin subunits on the internal surface of the MT protofilament, changing the tubulin conformation and interfering with protofilament disassociation (Xiao et al. 2006).

Oryzalin is a dinitroaniline class chemical that binds to α-tubulin (Morrissette et al. 2004). It can bind to both α-tubulin present in dimer form and α-tubulin in microtubules. This occurs in a polymer mass- and number-dependent manner, although polymerised tubulin has a much lower oryzalin-binding capacity than unpolymerised tubulin (Hugdahl and Morejohn 1993). Oryzalin binds to an area of α-tubulin close to the site of lateral protofilament interaction (e.g. below the N loop, Figure 1.1B). When it binds to free tubulin, oryzalin slows MT assembly by interfering with protofilament-protofilament binding, and when it binds to polymerised MTs, oryzalin destabilizes protofilament-protofilament interactions thus promoting MT disassembly (Hugdahl and Morejohn 1993; Morrissette et al. 2004).

Pronamide (also known as propyzamide), like oryzalin, is a powerful antimicrotubular chemical that interferes with MT polymerization. Pronamide belongs to the benzamide class of anti-MT chemicals, some of which bind to β-tubulin and are structurally distinct from dinitroanilines (Young and Lewandowski 2000; Robinson et al. 2004). There is relatively little known about how pronamide and other benzamides bind to β-tubulin to promote net MT depolymerization. However, pronamide is unable to depolymerise intact MTs but it appears to act in a manner similar to other MT disrupting chemicals in preventing MT polymerization by interfering with tubulin dimer formation or addition to the MT end (Akashi et al. 1988; Young and Lewandowski 2000; Robinson et al. 2004).

Low concentrations of taxol, pronamide, or oryzalin all have the same overall effect: reduced MT dynamics. MT disrupting chemicals present in low concentrations (e.g. 1 µM - 2 µM pronamide, 50 nM - 100 nM oryzalin) result in little net MT disassembly, but significantly lower MT growth and shrinkage rates with overall decreased MT dynamics (Nakamura et al.

2004). Low concentrations of taxol (e.g. 100 nM - 1 μM) also inhibit the growth and shortening of MT ends, resulting in increased MT stability without significantly affecting overall MT length (Derry et al. 1995).

When the concentration of these chemicals exceeds certain threshold levels, the effects are quite different. Taxol present in high concentrations (≥1 μM) stops nearly all MT polymerization/depolymerization, significantly increasing overall MT mass and MT bundling (Derry et al. 1995). High concentrations of oryzalin and pronamide cause cortical MT arrays to become disorganized, fragmented, and overall much thinner (Morejohn et al. 1987; Baskin et al. 1994). When present in sufficiently high concentrations, oryzalin and pronamide can cause the complete loss of intact plant MT arrays.

1.2 Low Temperature Stress

1.2.1 Low Temperature Stress in Plants

Low temperature stress is a major environmental stress that affects many aspects of plant biology. Low temperature limits everything from plant distribution to crop yield and product quality, and even the best crop varieties are restricted by short growing seasons and cold. All plants are susceptible to low temperature injury, but the mechanisms of injury and the temperature that causes these injuries vary considerably.

There are two distinct levels of low temperature stress, chilling and freezing stress. Chilling stress occurs when plants are exposed to low or non-freezing temperatures and is particularly common in plants of tropical and subtropical origin (Lyons 1973; Nick 2000). Biochemical reactions proceed at reduced rates at low temperatures and chilling sensitive plants are damaged by physiological dysfunctions caused by slower metabolic activity and metabolic imbalances (Lyons 1973). Chilling reduces membrane permeability and fluidity, and most chilling injuries are attributed to various types of membrane damage (Raison 1973; Sangwan et al. 2002). Chilling has also been shown to cause depolymerization of microtubules and actin microfilaments (Bartolo and Carter 1991; Wang and Nick 2001).

Freezing stress can take many forms in the plant and the degree of freezing injury a plant experiences is dependent on both the freezing tolerance of an individual plant as well as the characteristics of the freeze itself. The age, developmental stage and genotype of a plant, as well as the temperature of ice nucleation, cooling rate, minimum temperature reached, period of ice

exposure, thawing rate and post thaw conditions all affect the extent of injury that results from freezing (Palta et al. 1998).

At temperatures just below freezing, some plants survive through their ability to avoid water freezing in their tissues. In the absence of ice nucleators (substances that catalyse ice formation) some plants can supercool to temperatures below zero without freezing of the cell fluid. Once nucleation cannot be avoided, ice crystals form within the plant. Ice crystals form within the cell (e.g. in the cytosol) when plants are freezing sensitive or if the cooling rate is very rapid (e.g. 5°C/min), and this intercellular ice formation causes irreversible damage to internal and external membranes, resulting in cell death (Burke et al. 1976; Levitt 1980).

Cold hardy plants and plants cooled at slow rates are able to avoid intercellular ice formation in a variety of ways. Because of a lower solute concentration outside the cell, compared to within the cell, ice formation usually begins in the extracellular water. The formation of ice is further prevented by the accumulation of anti-freeze proteins and stable, soluble, non-reacting, low molecular weight organic compounds called compatible solutes (e.g. proline and glycinebetaine) (Guy 1990; Pihakaski-Maunsbach et al. 2001). These compounds increase the internal osmotic pressure and lower the freezing point of the cellular solution to prevent and/or control ice formation within the cell (Burke et al. 1976; Xin et al. 2007).

Ice can be limited to the extracellular space by the cell wall and PM. The cell wall can act as a barrier to reduce the propagation of extracellular ice while the PM prevents ice entry into the cell. PM disruption leads to ice entry into the cell causing lethal cellular disruption (Yamada et al. 2002). In some cases, ice accumulation can cause mechanical damage from adhesions forming between membranes and cell walls and intercellular ice (Levitt 1980; Steponkus et al. 1993).

Although extracellular ice itself rarely causes cell damage, freezing eventually results in cell damage due to freeze induced cellular dehydration. Formation of extracellular ice changes the cellular water potential and causes water to move down the chemical potential gradient and out of the cell, until a balance is reached between the external and internal water potential (Levitt 1980; Steponkus et al. 1993). Although the removal of water reduces the likelihood of cell freezing plants can loose up to 90% of active cellular water at -10°C and can generally only survive this extreme dehydration for short periods (Thomashow 1999). The severe dehydration within frozen cells results in other harmful cellular changes, such as protein denaturation,

increased reactive oxygen species and impaired membrane folding (Steponkus et al. 1993; Thomashow 1999; Wisniewski et al. 2003). Membrane dysfunctions caused by freeze-induced cellular dehydration are considered the primary cause of freezing injury in plants (Steponkus 1984). Membranes can be damaged by the formation of endocytic vesicles and expansion-induced lysis, or more severe dehydration can damage membranes because of irreversible interactions between membranes, loss of osmotic responsiveness, membrane lesions, and phase transitions (Browse and Xin 2001).

1.2.2 Cold Acclimation

Because plants are sessile organisms it is very important for them to be able to quickly adjust their biology to deal with changing environmental stresses. Various mechanisms have evolved to enable organisms to sense their external environment and eventually turn on the appropriate response. One of the best-studied plant stress responses and a key factor in determining the degree of freezing injury a plant incurs is cold acclimation (Levitt 1980; Thomashow 1999). Cold acclimation is a process through which plants acquire increased freezing tolerance upon exposure to low, non-freezing temperatures, usually between $0^{o}C$ - $6^{o}C$. Low temperature causes significant changes in the cell biophysics, and the acclimation process facilitates changes to plant cell structure, metabolism, and biochemistry that help a plant to efficiently operate under the new, colder conditions. The acclimation process is capable of increasing freezing tolerance significantly, for example, freezing at $-5^{o}C$ can kill non-acclimated rye while acclimated rye can survive temperatures down to about $-30^{o}C$ (Uemura et al. 1995). Most cellular processes are affected by cold acclimation, and although some of these processes are required for increasing plant freezing tolerance, others may merely change in response to the low temperature. A number of genes and cell processes that respond to cold acclimation have been described, yet there are many aspects of the cold acclimation process that have yet to be explained (Hannah et al. 2005).

1.2.2.1 Cold acclimation induced genetic and cellular changes

Studies have demonstrated that transcript levels of 45% of the Arabidopsis gene complement can change in response to low temperature (Chinnusamy et al. 2003; Zarka et al. 2003). Some of those genes that are responsible for affecting important changes in plant freezing tolerance during the acclimation process have been characterized. The C-repeat Binding Factor (CBF) dependent signalling pathway is a well characterized pathway containing many genes

important for cold acclimation and other stress responses (Figure 1.2B). Low temperature is believed to activate INDUCER OF CBF EXPRESSION (ICE) transcription factors, which stimulate the transcription of the *CBF* (a.k.a. *DEHYDRATION-RESPONSIVE ELEMENT-BINDING (DREB1)*) genes (Chinnusamy et al. 2003). *CBF* genes encode transcription factors that bind to the dehydration-responsive element/C-repeat (DRE/CRT) regulatory elements present in the promoter region of *COLD-RESPONSIVE* (*COR*) (a.k.a. *LOW TEMPERATURE INDUCIBLE (LTI*); *COLD INDUCIBLE* (*KIN*); *RESPONSIVE TO DESICCATION* (*RD*) and *EARLY DEHYDRATION INDUCIBLE* (*ERD*)) genes (Warren et al. 1996; Knight et al. 1999). Binding of CBF/DREB to the regulatory region promotes transcription of *COR* genes containing these elements. Inositol polyphosphates (InsP) and calcium ion (Ca^{2+}) levels increase following exposure to low temperatures and act as secondary messengers to promote the activity and expression of the other elements. Other genes involved in the pathway include *FIERY1* (*FRY1*) and *HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE 1* (*HOS1*) genes, which reduce stress signalling by negatively regulating InsP and ICE or upstream signalling elements, respectively (Figure 1.2B).

Other genes involved in the freezing process have been identified by mutational analysis. The *SENSITIVE TO FREEZING* (*SFR*) genes were identified due to a decreased ability of mutants to acclimate (Xin and Browse 1998). The *ESKIMO 1* (*ESK1*) gene was identified in a mutant that possessed constitutive freezing tolerance under both acclimated and non-acclimated conditions (Guy 1990; Browse and Xin 2001; Thomashow 2001; Livingston et al. 2007). ESK1 gene product controls transcription of a set of stress responsive genes, largely independent of genes regulated by cold acclimation and CBF, with more overlap with salt, osmotic stress and abscisic acid (ABA) induced genes (Xin et al. 2007).

Although many cold responsive genes have been identified, the biochemical activities of their products are poorly understood. However, the general function of these genes can be predicted, with cold responsive genes classified into groups that represent the variety of biochemical and physiological processes that are affected by the acclimation process. Growth cessation, enhanced antioxidative mechanisms, increased ABA concentration, changed membrane lipid composition, accumulation of soluble sugars, amino acids, cryoprotective and antifreeze proteins, and increased or decreased expression of other genes all help contribute to

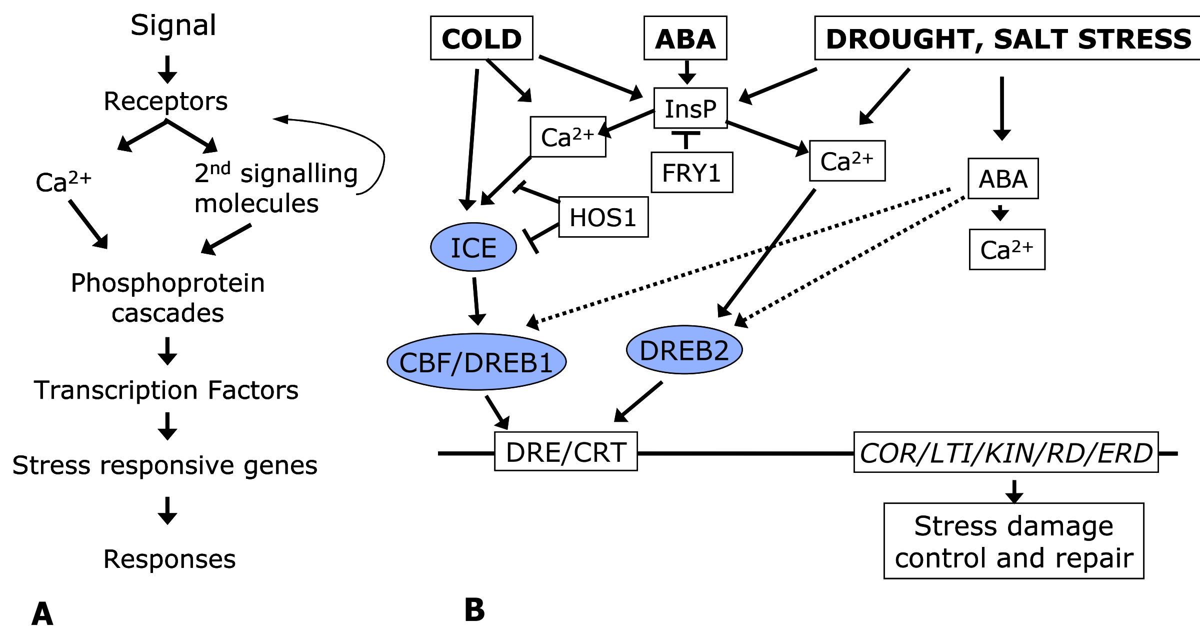

Figure 1.2. Abiotic stress signalling pathways. (A) Overview of general stress response pathway. Signal transduction begins when signals are perceived by receptors, which is followed by the generation of Ca^{2+} and secondary messengers. These signalling elements instigate phosphoprotein signalling cascades, leading to the activation of transcription factors and transcription of stress responsive genes which encode elements that ultimately increase the stress tolerance of the organism. (B) Pathways that activate DRE/CRT *cis* element containing genes. Cold, drought, salt stress, and ABA can activate DRE/CRT containing *COR* genes through stress and ABA inducible transcription factors (CBF/DREB1 and DREB2). The transcription factor ICE promotes CBF3 expression. Ca^{2+} and InsP are secondary messengers with multiple activators and effects on the signalling cascade. FRY1 negatively regulates InsP levels and stress signalling, and HOS1 appears to negatively regulate cold signalling by targeting ICE or upstream signalling components for degradation. Dashed arrows indicate the method of activation is unknown. Abbreviations are given in text. Image modified from Xiong et al. (2002).

increased freezing tolerance during cold acclimation (Guy 1990; Griffith et al. 1992; Pihakaski-Maunsbach et al. 2001; Uemura and Steponkus 2003).

Both acclimated and non-acclimated *esk1* mutants are characterized by high levels of proline and sugars, which may be wholly or partially responsible for the constitutive freezing tolerance of the mutant (Xin et al. 2007). *sfr4* mutants are overly sensitive to freezing, and have low levels of sugars and 18:1 and 18:2 fatty acids levels (Uemura et al. 2003). Overexpression of Arabidopsis CBF3 elevates proline and total sugars levels, indicating that *COR* genes may also be involved in regulating solute levels (Gilmour et al. 2000). ERD14 is a COR protein that protects the cell from damage incurred during rapid cooling, possibly by minimizing ice penetration from the extracellular space into the cell (Uemura et al. 2006). Extensin and EARLI1 are two types of low temperature responsive cell wall proteins that are important in protecting the cell during freezing stress via currently unknown mechanisms (Yamada et al. 2002; Zhang 2007).

Dehydrins are low temperature responsive hydrophilic proteins that can act as emulsifiers or chaperones to protect membranes and proteins from low temperature and dehydration induced damage (Close 1997). Many of the *COR* genes encode low temperature induced dehydrins (e.g. COR47, LTI30, RAB18, ERD10, and ERD14) which stabilize macromolecules during desiccation (Thomashow 1999; Kawamura and Uemura 2003). The expression of the *RAB18* dehydrin seems to be downregulated by ESK1, which may partially contribute to the increased freezing tolerance of *esk1* mutants (Kawamura and Uemura 2003).

Membrane changes occurring during acclimation help prevent or reduce freezing injury. In addition to the membrane protecting dehydrins, fatty acid desaturases, lipid transfer proteins, and a variety of membrane associated proteins act during the acclimation process to increase membrane cryostability (Kawamura and Uemura 2003). During the acclimation period the levels of proteins that control proteolysis, protect membranes against osmotic stress, enhance CO_2 fixation, and participate in other membrane repair mechanisms all increase within days (Uemura et al. 2006). There are many changes in lipid composition during cold acclimation: higher proportions of phospholipids, primarily unsaturated phospholipids, and relatively low levels of saturated phospholipids and sterols help increase membrane fluidity and lower the critical temperature of low temperature induced phase transitions (Nakayama et al. 2007). The best characterized COR protein, COR15, is important in preventing membrane freezing injury by

preventing chloroplast-PM associations and membrane phase transitions (Fowler and Thomashow 2002; Chinnusamy et al. 2003; Xin et al. 2007).

Abiotic stress stimuli are complex, however, different types of stresses activate similar responses and signalling pathways (Figure 1.2). Similar changes to gene expression and metabolism are induced by exposure to alternative dehydrative conditions that trigger common abiotic stress response pathways, including ABA stress signalling, mitogen-activated protein kinase (MAPK) cascades, and Ca^{2+} responses (Xiong et al. 2002). There are multiple pathways that contribute to increased plant freezing tolerance, and it is likely that single aspects of the stress condition can initiate multiple branches of signalling cascades. It is currently unclear whether any cold stress response pathways acts completely independently of others. There does appear to be both ABA dependent and ABA independent cold acclimation pathways in plants that regulate general and low temperature specific stress response pathways, respectively. Cross talk does occur between elements of these two pathways (e.g. both generate the same secondary messengers) (Xiong et al. 2002; Figure 1.2B).

1.2.2.2 General stress sensing

Before any stress-response pathway can be activated, the plant must somehow sense it is being exposed to the stress. All living organisms use various receptors exposed on the cell surface to perceive and process information about their environment, and plant cells should not be different. However, the elements responsible for sensing various stresses have not yet been clearly identified in plants, and identification of these sensors and receptors is made difficult by the genetic redundancy and complicated feedback and compensation mechanisms of plants (Kacperska 2004; Humphrey et al. 2007)

In plants, the cell wall (extracellular matrix) is the most external surface of the cell, and is therefore the first element to receive the stress signal and begin the signal transmission to the cell interior (Baluska et al. 2003). The cell wall is intimately associated with both the PM and cytoskeleton, and the close association between these elements is an essential feature that enables plant cells to effectively respond to extracellular signals (Cyr 1994; Sonesson and Widell 1998; Baluska et al. 2003). Communication across the cell wall-plasma membrane-cytoskeleton continuum is a characteristic feature of plant cellular mechanics, and this relationship facilitates the transmission of cell wall and PM modifications to MTs (Akashi et al. 1990; Nguema-Ona et al. 2007). The cell wall affects the stability of the underlying MTs, demonstrated by the

disassembly of MTs following disruption of the cell wall, and the existence of certain cell wall proteins (e.g. extensin, AGPs) that increase the cold stability of cortical MTs while other cell wall proteins (e.g. xyloglucan) causes MT disassembly and reorganization (Akashi et al. 1990; Takeda et al. 2002). MT integrity can affect properties of the PM; in animals and maize protoplasts MT depolymerization has been shown to increase PM fluidity (Dugas et al. 1989).

The continuum between MTs, the PM, and the cell wall is maintained under various stressful conditions. Even when the cell wall-PM connection appears to be disrupted when the membrane shrinks away from the cell wall during plasmolysis, the continuum is maintained. During plasmolysis, Hechtian strands, composed of plasmalemma, actin filaments and microtubules, link the cell wall to the PM (Buer et al. 2000). Hechtian strands are believed to be important in cell-to-cell communication and signal transduction from the cell wall, and interestingly, cold acclimation appears to increase their strength and flexibility (Buer et al. 2000). While the close association between cell wall, PM, and MTs is essential for responding to extracellular signals, this close association and the convolution of this relationship also makes it difficult to identify which cellular element(s) is responsible for sensing low temperature and complicates the study of early events involved in the low temperature signalling pathway (Xiong and Zhu 2001; Baluska et al. 2003).

1.2.2.3 Low temperature sensing and signalling elements

All abiotic stresses are thought to follow the same basic signal transduction pathway (Figure 1.2A). After signal receptors are stimulated by the stress there is a transient increase in levels of signalling molecules and cytosolic calcium (Ca^{2+}). Ca^{2+} levels rise because of the opening voltage-gated, receptor operated, and ligand messenger-sensitive Ca^{2+} channels (Xiong et al. 2002). Inositol phosphates (InsP) and reactive oxygen species (ROS) are secondary messengers activated by cold and other stresses that can also modulate their own levels and stress-induced Ca^{2+} influx (Xiong et al. 2002). The increased Ca^{2+} levels and other secondary messengers initiate protein phosphorylation cascades (kinase cascades), which signal the activation of transcription factors that control specific groups of stress-regulated genes. These stress-regulated gene products may also be involved in generating regulatory molecules themselves, like the plant hormone abscisic acid (ABA), which in turn can initiate a second round of signalling (Nick 2000). The transduction of these intracellular signals may involve

physical and/or chemical changes that occur to the signalling intermediates, ranging from phosphorylation to movement of the elements throughout the cell.

The low temperature response pathway involves these same elements. Although the cellular element(s) that sense low temperature and initiate the first stages of low temperature signalling are not known, two of the earliest described low temperature responses are decreased membrane fluidity and remodelled cytoskeleton (Sangwan et al. 2002). It has been proposed that the opening of Ca^{2+} channels and Ca^{2+} influx occurs immediately following membrane and cytoskeleton changes, which in turn triggers protein kinases and cold-specific MAPK cascades, leading to the activation of cold-induced genes and the acquisition of freezing tolerance (Murata and Los 1997; Sangwan et al. 2002; Wasteneys and Galway 2003). Multiple pathways leading to expression of the same stress-activated transcription factors complicates the pathway, as shown in Figure 1.2.

As mentioned, the cellular element responsible for sensing low temperature is not known, and details of the early low temperature-signalling pathway are missing. However, much of the later pathway is known and/or can be pieced together, and a few elements with the potential to mediate linkages between the cell wall-PM-MTs and participate in the process of sensing and responding to low temperature signals have been suggested.

1.2.2.3.1 Plasma membrane. Because of its position on the outer cell periphery and the structural changes observed after short term exposure to low temperatures, the PM was long believed to be the primary site of temperature change perception in plants (Murata and Los 1997). Temperature change rapidly and reversibly affects the fluidity or viscosity of membranes: high temperature increases membrane fluidity and low temperature increases membrane viscosity. Membrane rigidification is one of the first visible responses to low temperatures and reports indicate that it is a necessary element of the low temperature signal transduction pathway (Örvar et al. 2000; Sangwan et al. 2001). Artificially reducing membrane fluidity in *Medicago sativa* cells and *Brassica napus* plants caused a modest increase in levels of cold-induced gene transcripts as well as a moderate increase in the cells freezing tolerance, while artificially increasing membrane fluidity reduced expression of cold-induced genes and obstructed cold acclimation (Murata and Los 1997; Sangwan et al. 2002). Because there are so many changes that occur to the PM during low temperature exposure, even if the membrane rigidification is the

primary means of sensing low temperatures, the specific membrane component that is responsible for transmitting these changes as a low temperature signal is not known

1.2.2.3.2 Calcium channels. Transient increases in cytosolic calcium (Ca^{2+}) levels are an important activity involved in many signal transduction pathways. Ca^{2+} influx is essential to low temperature signalling in plants, and it occurs very rapidly upon low temperature exposure in all plants studied (including Arabidopsis and alfalfa) (Sangwan et al. 2002). Artificially inducing Ca^{2+} influx activates cold-inducible genes in the absence of cold while preventing Ca^{2+} influx using calcium chelators or calcium channel blockers prevents the accumulation of cold-induced gene transcripts, interrupts the ability of the plant to cold acclimate, and inhibits cold-induced protein phosphorylation (Knight et al. 1996; Sangwan et al. 2001; Xiong and Zhu 2001). There are different sources of Ca^{2+} influx following temperature stress, but the primary source of cold-induced Ca^{2+} influx appears to be cell wall calcium stores (Sangwan et al. 2001).

It was recently shown that the primary sensor of mechanical stimulation is a stretch-activated Ca^{2+}-permeable membrane channel that also controls the entry of Ca^{2+} into the cell (Nakagawa et al. 2007). It is possible that there are similar Ca^{2+} ion channels that directly respond to other stresses.

1.2.2.3.3 Kinase cascades. Phosphorylation/dephosphorylation is the most common form of rapid signal transduction, and kinase cascades are thought to be central to cold acclimation pathways (Monroy et al. 1993; Monroy et al. 1998; Sangwan et al. 2001). Protein phosphorylation can lead to expression of cold-induced genes while treatment with kinase inhibitors decreases cold-induced gene expression (Kawczynski and Dhindsa 1996; Monroy et al. 1998; Sangwan et al. 2001). Kinases are important in sensing various stresses; kinase sensor and receptor systems are the most common stress sensing systems found from prokaryotes to fungi and primitive animals (Urao et al. 1999; Novikova et al. 2007). Similar systems of stress sensing and signalling appear to occur in plants, for example, Arabidopsis salt and osmotic stress sensing appears to involve a salt stress responsive receptor histidine kinase (AtHK1) (Urao et al. 1999; Humphrey et al. 2007).

There are multiple types of kinases found in plants. One well-characterized group of kinases that are activated during cold acclimation are the mitogen activated protein kinases (MAPKs) (Samaj et al. 2004). Other kinases proposed to be involved in initiating low temperature stress signalling include lectin receptor kinases, wall-associated kinases (WAKs)

and proline-rich extensin-like receptor kinases (PERKs) (Humphrey et al. 2007). These proteins are located at the PM with an external domain embedded within the cell wall and an intracellular kinase domain, making them ideally localized for sensing and signalling, and quickly and directly transmitting phosphorylation signals through the plant (Humphrey et al. 2007).

1.2.2.3.4 Microtubules. Previous reports suggest that the cytoskeleton may act as a low temperature sensor in plants (Thion et al. 1996; Abdrakhamanova et al. 2003). As previously mentioned, changes to the cell wall and plasma membrane (including the rigidification of membranes that is important to the cold acclimation process), cause subsequent changes to MTs. MT disassembly has been connected to cold induced gene expression and elements of the low temperature signalling pathway, with disassembly possibly required for efficient cold acclimation (Kerr and Carter 1990; Bartolo and Carter 1991; Örvar et al. 2000).

MT activity can mediate Ca^{2+} channel opening since disassembly of MTs results in increased activity of voltage-dependent Ca^{2+} channels (6-10 fold increases) (Thion et al. 1996). The relationship is more complex, however, as Ca^{2+} influx has also been shown to induce the depolymerization of MTs (Breviario and Nick 2000). It is thought that Ca^{2+} may regulate MT dynamics by calmodulin dependent MT-MAP interactions or by acting on tubulin C-termini. In maize, cleavage of this end-domain renders MTs resistant to both Ca^{2+} and cold induced disassembly (Bokros et al. 1996; Nick 2000). It has been proposed that in response to low temperatures, MTs disassemble, Ca^{2+} channels activate, and intracellular Ca^{2+} levels transiently rise and proceed to trigger downstream events in the cold acclimation process (Nick 2000).

Phosphorylation is also linked to MT stability. Activation and inhibition of kinases and phosphatases affect MT organization (Baskin and Wilson 1997; Naoi and Hashimoto 2004). MAPKs associate with MTs in many cell types; some plant MAPs (the kinesins) are able to activate MAPKs, and either tubulin or MAPs are phosphorylated by MAPKs affecting cortical MT organization and dynamics (Koontz and Choi 1993; Cyr 1994; Samaj et al. 2004).

The relationship between low temperature stress and MTs is more complex than described here, and these interactions will be described in more detail in Chapter 3 of this text.

1.2.2.3.5 Cell wall proteins. Some structural cell wall proteins have been proposed to be involved in sensing low temperature. Besides the cell wall kinases previously mentioned, two other protein types, the extensins and arabinogalactan proteins (AGPs), are good candidates for linking the extracellular matrix to the inside of the cell and MTs and mediating stress responses.

Extensins are a group of well studied hydroxyproline-rich glycoproteins implicated in nearly all elements of plant growth and development, including cell division and differentiation, cell elongation cessation, and biotic and abiotic stress responses (Showalter 1993; Yoshiba et al. 2001; Roberts and Shirsat 2006). Both the presence and cross-linking of extensins obstruct cell elongation. Extensin proteins are cross-linked in response to wounding to strengthen the cell wall in what is believed to be a rapid defence response (Roberts and Shirsat 2006). The activity of extensin proteins causes increased cold stability of cortical MTs, while disruption of extensins causes MT disorganization (Akashi et al. 1990). It has been speculated that cortical MTs are cross-linked to the cell wall via a cross-bridge-transmembrane protein-extensin system and this cross-linking is enhanced by stress responsive elements such as hydrogen peroxide (Akashi and Shibaoka 1991). Another stress responsive proline-rich cell wall protein, EARLI1, behaves similar to extensin proteins. It contains a putative signal peptide and plasma membrane interacting domain and is known to be important for freezing survival, possibly through a similar mechanism as extensins (Zhang 2007).

Arabinogalactan proteins (AGPs) are cell wall proteoglycans that are involved in linking the cell wall - PM and MTs, and participate in controlling the flow of information between these elements (Nguema-Ona et al. 2007). These wall proteins can be anchored in the plasma membrane by means of a glycosyl phosphatidylinositol (GPI) anchor, which is added to the C-terminal end of certain classes of AGPs (Borner et al. 2002). AGP disruption/precipitation causes cortical MTs to become disorganized and separate from the plasma membrane, and induces gaps between the cell wall and membrane, suggesting that AGPs may be important elements in connecting these structures (Nguema-Ona et al. 2007). AGP can be induced by ionic changes and mechanical stimulation, and may be involved in stress sensing or signalling cascades (Humphrey et al. 2007). Disruption of MTs via the disruption of AGPs causes Ca^{2+} influx. While this Ca^{2+} influx does not cause the MT disassembly, it is unclear if the AGP disruption itself or the subsequent MT disassembly is responsible for the Ca^{2+} influx (Nguema-Ona et al. 2007).

The GPI anchor on AGPs can be cleaved by phospholipase C (PLC) or phospholipase D (PLD), changing the AGPs from PM anchored to extracellular proteins (Borner et al. 2003). PLD has been shown to link cortical MTs to the plasma membrane (Hong et al. 2008), and PLD controls cytoskeleton stability and linkage to the plasma membrane in a Ca^{2+}dependent and stress (including oleic acid and H_2O_2) activated manner (Gardiner et al. 2001; Dhonukshe et al.

2003; Zhang et al. 2003; Sedbrook 2004; Cai et al. 2005). PLD is important in stress signalling cascades: it promotes drought stress tolerance by promoting stomatal closure at early stages of exposure, but after prolonged drought stress it causes membrane disruption (Hong et al. 2008). These effects resemble changes that occur to other cellular elements that appear to be important to signal sensing, but cause cell damage if not discontinued after a short period (e.g. ROS signalling and cell damage).

1.3 Confocal Microscopy for Microtubule Analysis

Green fluorescent protein (GFP) was originally derived from the jellyfish *Aequorea victoria*. When exposed to blue light, GFP fluoresces green, but the original jellyfish GFP has been modified in various ways to create fluorescent proteins (FPs) that can emit bright fluorescent signals at various well defined light spectrums, ranging from red to cyan (Stauber et al. 1998). A number of enhanced FP variants have been created that are optimized for plant studies (Davis and Vierstra 1998; Stauber et al. 1998). GFP can be used as an in vivo tag, when fused to other proteins it becomes possible to observe them within living cells. GFP fusion proteins have revolutionized fluorescence microscopy and our understanding of the structure and function of living cells (Brandizzi et al. 2002; Yuste 2005), and fluorescent microscopy gave us our first understanding of cytoskeleton dynamics and organization (Lloyd 1987).

Microtubule-binding proteins, tubulin, and plus-end-tracking proteins are all suitable for labelling microtubules in plant cells, and many types of cytoskeleton fluorescent labelling exist (reviewed in Yoneda 2007). While each type of reporter protein partner has their own advantages and disadvantages, FP-tubulin fusion proteins appear to be the best way to label entire cellular MT arrays. There are some side effects that can accompany transgenic FP labelled tubulins, such as the potential for increased MT polymerization and suppressed endogenous tubulin synthesis when expressed at high levels. When FPs are added to the α-tubulin N-terminus or β-tubulin C-terminus MT polymerization is affected significantly (Ueda and Matsuyama 2000; Abe and Hashimoto 2005; Yoneda et al. 2007).

These problems seem to be avoided in the transgenic Arabidopsis line GFP:TUB6 (Abe and Hashimoto 2005). This line contains a soluble-modified red-shifted version of GFP (smRS-GFP) (Davis and Vierstra 1998) fused to the N-terminus of β-tubulin TUB6 expressed under the control of the constitutive Cauliflower Mosaic Virus 35S promoter. GFP:TUB6 proteins are

present in this line at 20-30% of endogenous tubulin levels, and fluorescently label the MTs in aerial tissues, with the best expression observed in cells of the hypocotyl region. In this line the fluorescent protein is constitutively expressed, yet the plants are indistinguishable from wild type plants in development and morphology (Abe and Hashimoto 2005). This is in contrast to other GFP-tubulin lines, including TUA6:GFP, where constitutive expression affects MT assembly and causes helical twisting of plant tissues (Abe and Hashimoto 2005).

One disadvantage of tubulin labelling that is not avoided in GFP:TUB6 is the difficulty differentiating individual MTs and determining the thickness of MT bundles. Unfortunately, the resolution capabilities of fluorescence microscopy do not permit precise resolution of individual MTs within bundles, and the number of MTs cannot be precisely counted using these techniques. MT bundles must be differentiated from singletons and thinner MT bundles by the increased brightness and thickness of large MT bundles (Mouriño-Pérez et al. 2006).

1.4 Objectives

The main objective of this thesis was to increase our understanding of how MTs may be involved in the cold stress response in Arabidopsis. This involved visualizing how various low temperature conditions affect MT organization in Arabidopsis, examining the importance of MT dynamics in cold acclimation, and examining if MT disassembly is able to increase freezing tolerance in the absence of the low temperature signal. This thesis also included a study aimed to identify Arabidopsis microtubule associated proteins that are likely to be involved in the plant cold acclimation process.

CHAPTER 2
LOW TEMPERATURE STRESS, COLD ACCLIMATION AND MICROTUBULE ORGANIZATION

2.1 Introduction

Low temperature is a major constraint to crop productivity in plants around the world, particularly in temperate regions. There are two distinct levels of low temperature stress that affect plants, chilling stress occurs at low but non-freezing temperatures and freezing stress occurs after exposure to sub-zero temperatures. Plants which are tolerant to chilling temperatures are able to avoid damage caused by the low temperature induced slowed metabolism and growth rate and reduced membrane permeability and fluidity (Raison 1973; Sangwan et al. 2002). Freezing damage occurs as a result of ice formation within a plant, and plants that are able to survive freezing temperatures do so by avoiding or tolerating this ice formation. Freezing can damage plants by direct membrane disruption, damaged protein structure, increased levels of harmful reactive oxygen species (ROS) or impaired membrane function caused by the cellular dehydration that accompanies the ice formation (Steponkus et al. 1993; Thomashow 1999; Wisniewski et al. 2003).

In many species, there is a close correlation between chilling sensitivity and the critical temperature that induces MT disassembly (Bartolo and Carter 1991; Wang and Nick 2001). For example, in chilling sensitive species, such as tobacco and cucumber, MTs disassemble at temperatures between 0-4°C, which causes significant damage to the plant (Nick 2000). Antimicrotubular drugs applied during chilling significantly accelerate and enhance this tissue damage (Rikin et al. 1980). Treatments that decrease general plant cold hardiness, such as the hormone gibberellic acid (GA), also increase MT chilling sensitivity (e.g. MTs depolymerise more readily in response to low temperatures) (Rikin et al. 1980; Akashi and Shibaoka 1987). In plants that can tolerate chilling stress, MT arrays can be maintained at sub-zero temperatures, and intact MTs have even been observed in plants subjected to temperatures that cause damage to the plant. For example, in non-acclimated winter rye that has an approximate freezing

tolerance of -5°C, intact MTs were observed at -10°C (Pihakaski-Maunsbach and Puhakainen 1995).

Cold acclimation is a process by which plants increase their freezing tolerance following exposure to low but non-freezing temperatures. For this process to occur, plants must first sense the reduction in temperature and turn on stress induced signalling cascades (such as kinase cascades and Ca^{2+} signalling). The cellular element(s) responsible for sensing low temperatures and initiating the signalling are unknown. Signalling cascades activate transcription factors and changes to low-temperature gene expression occur that ultimately enable plants to adapt to the stress and withstand or prevent freeze-induced damage (Thomashow 1999). Accumulation of compatible osmolytes and altered membrane lipid composition are two ways that acclimation decreases freezing damage, thus increasing plant cold hardiness (Browse and Xin, 2001).

Because of the apparent close correlation between MT stability and plant cold hardiness in a number of species, it seems reasonable to expect that cold acclimation brings about increased freezing tolerance in part by increasing MT stability. This is supported by the fact that acclimating temperatures cause changes in tubulin gene expression, and tubulin isotypes that appear to be more resistant to cold-induced depolymerization are expressed at higher levels during low temperature exposure (Abdrakhimova et al. 2003; Chu et al. 1993). However, increasing MT stability using the MT stabilizer taxol consistently lowers the acclimating ability of plants (e.g. rye, spinach, and canola), and this has been interpreted to mean that acclimation requires MT depolymerization (Bartolo and Carter 1991; Kerr and Carter 1990; Örvar et al. 2000).

The involvement of MT disassembly in the acclimation process is currently unclear. There are many elements of the general plant stress response associated with MT activity. Decreased membrane fluidity and the influx of Ca^{2+} from the cell wall into the cytosol are two of the earliest responses to low temperature stress, and MT disassembly is linked to both of these activities (Dugas et al. 1989; Thion et al. 1998). Cell wall disruption and plasma membrane rigidification both promote MT disassembly, while MT disassembly can increase membrane fluidity (Dugas et al. 1989; Akashi et al. 1990; Örvar et al. 2000). Some have proposed that the increased diffusion of membrane lipids and proteins caused by MT destabilization contributes to increased low temperature tolerance (Dugas et al. 1989; Mizuno K 1992). MT polymerization/depolymerization appears to control the activity of Ca^{2+} channels (Thion et al.

1998) while Ca^{2+} influx has also been shown to induce MT depolymerization (Breviario and Nick, 2000; Nick, 2000). Phospholipase D (PLD) may physically link cortical MTs and the plasma membrane, and PLD activity is linked to stress signal transduction, cytoskeleton rearrangement, and detachment of MTs from the plasma membrane (Gardiner et al. 2001; Dhonukshe et al. 2003; Sedbrook 2004; Cai et al. 2005). Additional links include kinase/phosphatase activity influencing the cold stability of MTs (Sangwan et al. 2001), abscisic acid (ABA) influencing cortical MT organization (Shibaoka 1994) and ROS promoting MT fragmentation in vitro (Xu et al. 2006).

Two studies have recently attempted to decipher how MT disassembly and/or reassembly are involved in the plant cold acclimation process via this obviously very complex relationship. The first study found that stabilizing MTs using taxol prevented cold induced gene transcript accumulation, while depolymerizing MTs with oryzalin induced expression of cold tolerance genes without affecting plant cold hardness (Sangwan et al. 2001). The authors proposed that MTs must disassemble to initiate the cold acclimation process but reassembly is needed for plants to actually become tolerant to the cold.

The second study examined the relationship between MT disassembly and cold hardiness in roots of three winter wheat cultivars (Abdrakhamanova et al. 2003). In the cold tolerant wheat cultivars, acclimation treatments caused rapid (< 1 d) and transient MT disassembly followed by re-forming, but no MT reorganization occurred in the cold sensitive cultivar. When Abdrakhamanova et al. (2003) treated these same cultivars with high concentrations of the MT depolymerizing chemical pronamide; root growth and cold tolerance were affected in a similar manner to an acclimation treatment. Based on these results the authors concluded, “partial MT disassembly was sufficient for efficient induction of cold hardiness.”

Because of the large difference in MT response to low temperature stress, it is unexpected that all of the wheat cultivars examined acclimate to similar extents. Freezing tolerance (as measured by LT_{50}, the lethal temperature for 50% of plants) was -18.6°C for the less tolerant cultivar (Bezostaya 1) cold acclimated for 3 weeks, while the ‘moderately’ freezing tolerant cultivar (Mironovskaya 808) had a freezing tolerance of -20.8°C under the same conditions (Vitamvas 2007). If such a significant difference in MT response is observed among different cultivars of the same species that differ in freezing tolerance by less than a few degrees

Celsius, it would be interesting to examine the effect of acclimating temperatures on MTs from different species that vary significantly in their ability to acclimate.

The model plant Arabidopsis is widely used for studying cold acclimation and freezing injury and is able to cold acclimate to a maximum LT_{50} of approximately -10°C after 1 wk at optimum conditions (Thomashow 1999, 2001; Kawamura and Uemura 2003). In the present work we examined the changes that occur to MT organization in Arabidopsis during cold acclimation and other low temperature treatments, and aimed to determine the relationship between MT dynamics and organization and cold hardiness in this model plant capable of a moderate degree of cold hardiness.

2.2 Materials and Methods

2.2.1 Plant Material and Growth Conditions

Arabidopsis thaliana (ecotype Columbia) seeds were surface sterilized with 70% ethanol followed by 30% bleach and plated on ½ MS medium (Murashige and Skoog 1962) (pH 5.7) containing 1% sucrose and 0.8 or 1.5% Phytagar (Gibco Invitrogen, California) on round 140 mm diameter Petri dishes. Seedlings were grown at 22°C with a 16 h photoperiod, 80 $\mu mol/m^{-2}s^{-1}$ light intensity. After 2 weeks (wk) of growth, test plates were transferred to a cold chamber (4°C, 12 h photoperiod) for acclimation treatment. Cold shocks were administered either as treatment at -6°C for 4 h, or treatment directly on ice (0°C) for time periods ranging from 0.25 h - 24 h.

The transgenic Arabidopsis (ecotype Columbia) line expressing GFP:TUB6, kindly provided by Takashi Hashimoto (Nara Institute of Science and Technology, Japan), was used in Confocal microscopy experiments. GFP:TUB6 seedlings were sterilized and grown as per conditions described above, but sterilized seeds were plated on vertically oriented 120 x 75 x 15 mm plates.

2.2.2 Chemical Treatments

Stock solutions of Taxol™ (paclitaxel, Sigma), pronamide (propyzamide, Supelco) and oryzalin (Supelco) were prepared by dissolving the chemicals in dimethyl sulfoxide (DMSO, Sigma) at concentrations to ensure the final DMSO concentration in the culture medium remained below 0.1%. For long term, low concentration chemical treatments, wild type (WT) and GFP:TUB6 Arabidopsis seeds were plated and grown for 2 wk on MS media (as described

previously) supplemented with taxol (0.3 - 6 µM), pronamide (1 - 6 µM), oryzalin (0.05 - 0.5 µM), or DMSO (<0.1%, control).

To observe the effects of short term treatment with high concentrations of MT disrupting chemicals WT and GFP:TUB6 seedlings sown on standard ½ MS medium were transferred to media containing pronamide or oryzalin (6 - 20 µM) for time periods ranging from 0.25 h - 24 h, with some treatments followed by a 12 - 24 h recovery period on standard MS medium. GFP-TUB6 seedlings were transferred to chemical containing media after 3 - 5 days of growth, while WT seedlings were transferred after 3 wk of growth.

2.2.3 Confocal Microscopy

Confocal imaging was performed using a Zeiss META 510 laser scanning Confocal microscope (www.zeiss.com) with either a Zeiss 25x Plan-Neofluor NA 0.8 multi-immersion or a 40x Plan Apochromat NA 1.4 water immersion objective lens. GFP was excited with the 488 nm wavelength of an argon laser (at 8% power output) with emission collected by the ChS1 518-561 nm filter.

Four-six day old GFP-TUB6 seedlings were subjected to chemical treatments or acclimating temperatures (4°C) for time periods ranging from 0.25 h to 2 wk. Immediately following treatments the seedlings were carefully transferred to slides, a coverslip was gently overlaid and epidermal cells from the hypocotyl region of the plant were scanned to observe MT organization. For observations of low temperature treated plants, slides were pre-cooled and images recorded within a short time (10 min) of transfer to the microscope. For quantitative estimation of the effect of acclimation temperature on MTs at least 70 cells from 30 different plants were analyzed for each time point. The effects of chemical treatments and cold shock treatments on MTs were much less variable, and approximately 40 cells from 10 plants were examined for each of these treatments. Each set of treatments and observations were preformed in at least two experiments.

2.2.4 Electrolyte Leakage Freezing Tests

The aerial tissue from chemically treated or WT two-week-old Arabidopsis plants were used for freezing tests. Immediately after dividing the plants at the lower hypocotyl the green tissue was carefully placed on top of moistened filter paper discs horizontally positioned in 40 ml glass tubes; tissue from three plants was added to each tube to represent one replicate. Six – nine replicates were used per treatment type. The tubes were placed in an ethylene glycol bath

programmed to hold the temperature at -2°C before lowering the temperature 1°C per h. After 1 h at -2°C the tubes were ice nucleated by quickly touching the filter paper disc with a dry ice cooled spatula.

At each test temperature three treatment tubes were removed from the bath and allowed to thaw overnight at 4°C. The test temperatures were chosen to span from 0 to 100% injury, which ranged from -2°C to -6°C in non-acclimated plants and -2°C to -12°C in cold acclimated plants.

Thawed tissues were transferred to 40 ml tubes containing 15 ml distilled water and 0.05% Tween-20 and shaken at room temperature for 4 h to facilitate leakage of electrolytes out of the cells. Electrical conductivity (EC) was measured using a conductance meter (Orion 150A, Thermo). The leaf samples were then frozen at -20°C overnight to release total electrolytes, and were thawed and again shaken at room temperature for 4 h to release electrolytes. The % EC was calculated as electrical conductivity after freezing treatment / total electrical conductivity x 100. The temperature at which 50% EC occurred was determined to be the freezing tolerance (LT_{50}) (e.g. the temperature which 50% of the cells were killed) (Griffith and McLntyre 1993) (Ueda and Matsuyama 2000). The calculated LT_{50} values were expressed as means of three independent experiments. Two sided student's t-tests were performed to determine if any of the observed variations were significant ($p \leq 0.05$).

2.3 Results

2.3.1 Microtubule Response to Low Temperatures

MTs were observed as green-fluorescent fibres detectable beneath the cell walls of living cells in GFP:TUB6 Arabidopsis seedlings. The length and organization of these MTs was examined in cells throughout the plants, and a large amount of variability was seen with regards to MT orientation, which appeared to relate partially to cell shape and state of elongation. Much of this variability was removed when only plants of the same maturity (~5 days post germination with approximately 4 mm long hypocotyls) and cells from the same region of the hypocotyl were examined. In general, control cells contained MTs that were long, usually extended across the external cell cortex primarily in parallel to each other in transverse or oblique orientation (Figure 2.1A and 2.2).

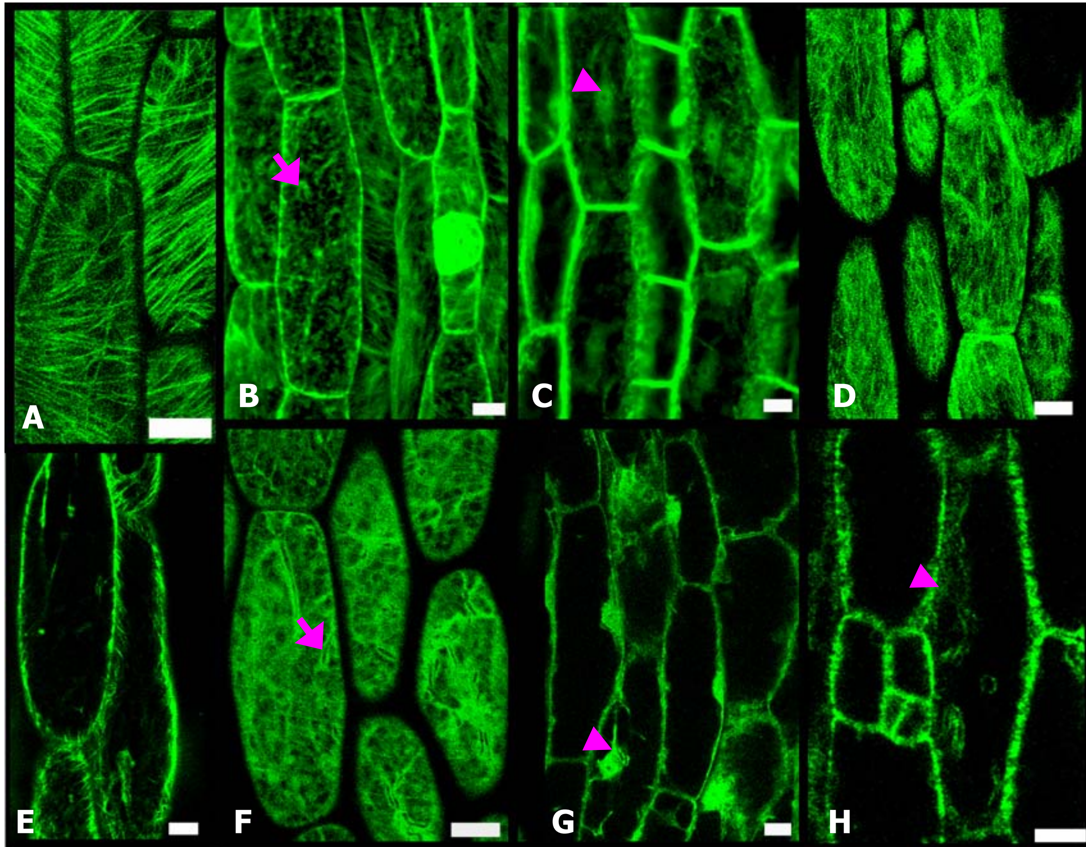

Figure 2.1. Response of non-acclimated Arabidopsis GFP:TUB6 MTs to freezing shock. (A and E) Cells of untreated control plants showing (A) long, organized MTs and (E) few cytoplasmic strands of tubulin. Disassembled MTs and intranuclear tubulin fluorescence after exposure to (B-C) 4 h freeze shock at -6°C, and (F-G) 24 h exposure to 0°C. Reassembly of MTs and loss of intranuclear fluorescence after a 4 h period at 25°C following (D) 4 h freeze shock at -6°C and (H) 24 h exposure to 0°C. A, B, D, and F, Z-stacked images of the region of cortical MTs at cell surface; C, E, G and H interior section of hypocotyl cells through region of nucleus. Arrows indicate disk-like structures and fluorescent granules, arrowheads indicate nuclei. Images are representative of approximately 40 cells observed for each treatment. Scale bars = 10 µm.

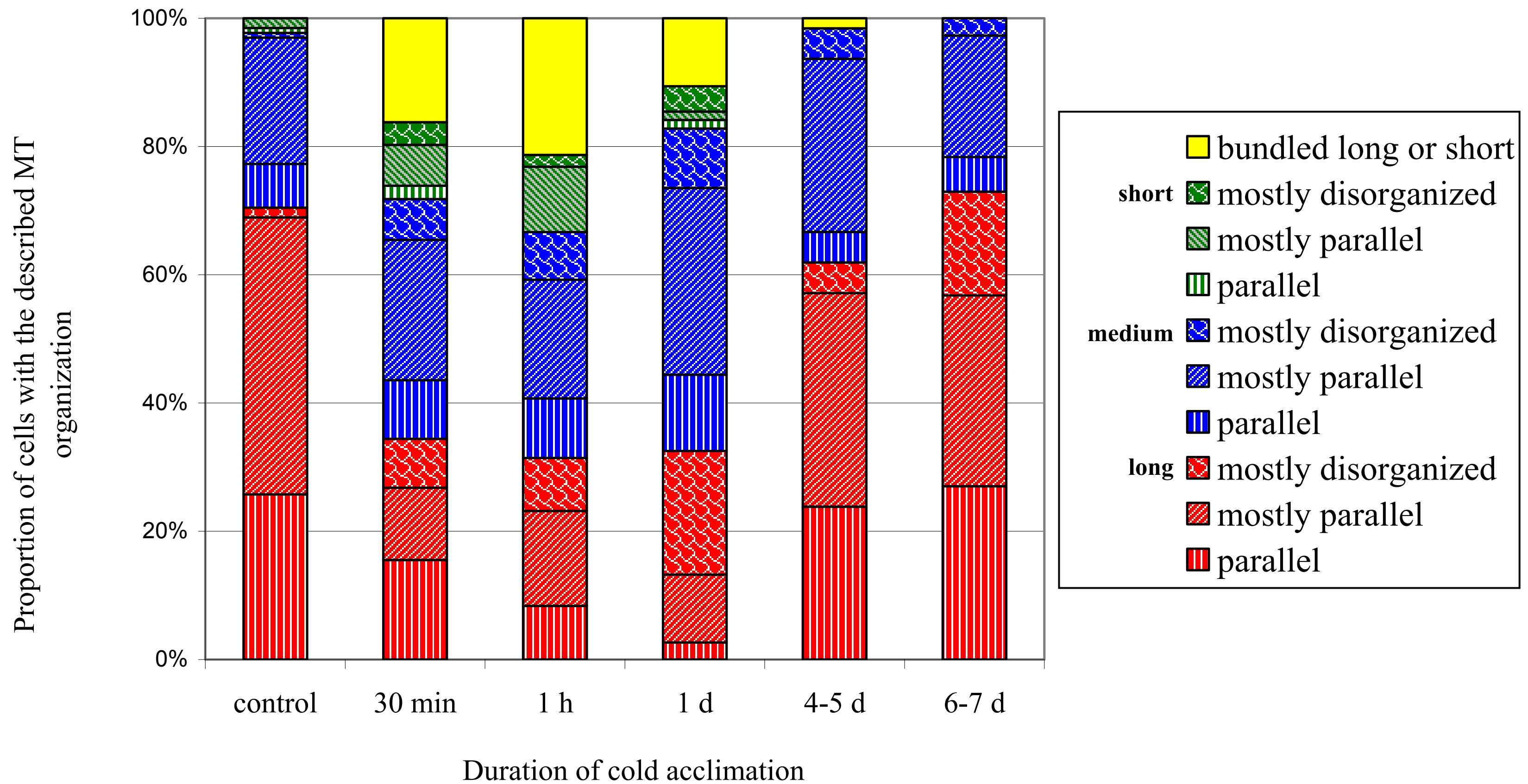

Figure 2.2. Distribution of cortical MT array organization in GFP:TUB6 Arabidopsis seedlings subjected to cold acclimation at 4°C for the shown periods of time. The cortical MT arrangement of individual cells was determined to be organized into either highly parallel, mostly parallel or disorganized arrays, and the predominant length of MTs in the cells were determined to be either long (primarily extending fully across the cell), short (primarily <5 µm) or mid-length . The category bundled long or short indicates that the cell contained an approximately equal proportion of long, thick MTs and thin, fragmented MTs. Over 70 cells from over 30 seedlings were examined to generate data for each treatment point.

Plants subjected to a cold shock at temperatures at or below zero degrees Celsius underwent dramatic changes to MT organization (Figure 2.1). Short periods (<2 h) at freezing temperatures caused MTs from most cells to become significantly shortened and disorganized, and completely lose their organized array structure (Figure 2.1B). After prolonged periods of exposure (>1 day (d)) to freezing temperatures (when the plants were able to cool to the external temperature) intact MT arrays were abolished (Figure 2.1F). Under freezing conditions some cells were able to maintain their MT arrays, and these apparently cold stable MTs were often organized in parallel, oblique or transverse arrays (Figure 2.1B). However, more commonly the fine strands of MTs were replaced with fluorescent accumulation in diffuse and bright patches of different shapes and sizes throughout the cytoplasm (Figure 2.1B and F).

Tubulin fluorescence also collected in the plant nucleus (but not nucleolus) of many cells in cold shocked plants (Figure 2.1C and G). This passage of tubulin through the nuclear membrane occurred in cold shocked plants whether or not the cold shock was sufficient to cause plant death. However, longer and more severe low temperature treatments resulted in a higher proportion of cells with intranuclear tubulin. Both the MT disassembly and intranuclear tubulin fluorescence were reversible in plants able to recover from the cold shock; upon plant rewarming MTs reformed and tubulin rapidly (within 30 min) dissipated out of the nucleus (Figure 2.1D and H).

2.3.2 Microtubule Response to Acclimating Temperatures

Because acclimation temperature treatments do not affect all cells and all plants equally, the MT organization of multiple cells and plants were examined and classified based on the MT length and general order. The effect of acclimating temperatures on cortical MT organization was examined in WT Arabidopsis seedlings subjected to acclimating temperatures (4°C) for time periods ranging from 0.5 h to 1 wk, and Figure 2.2 gives an overview of MT organization throughout the period of low temperature treatment.

Following short periods at acclimating temperatures the cortical MT array tended to break up into shorter lengths and undergo a general disorganization. After just thirty minutes at 4°C the proportion of cells containing long MTs was reduced to half that of control cells (Figure 2.2 and 2.3B). Approximately 30% of cells from short-term (0.5 - 1 h) low temperature treated plants were characterized by thin and fragmented MTs (Figure 2.3C). However, half of these

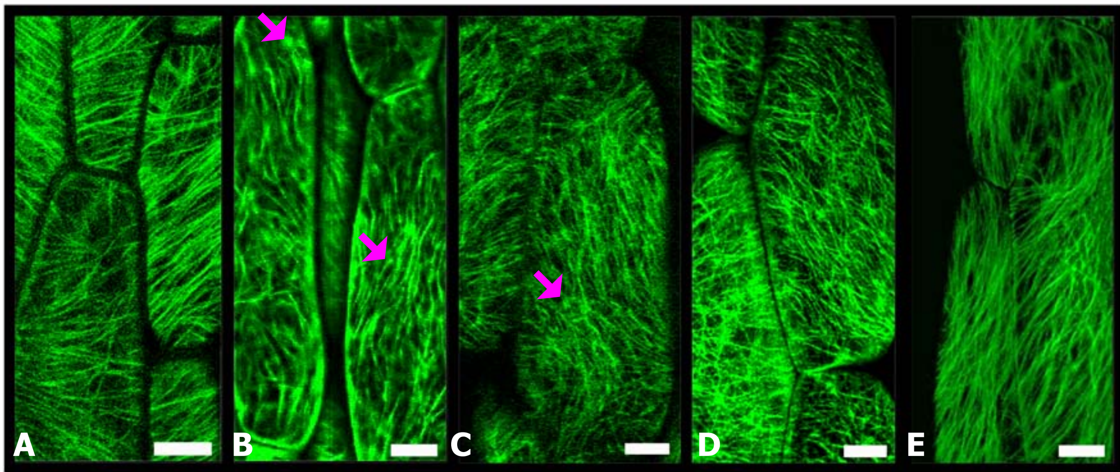

Figure 2.3. Confocal micrographs of cortical MTs from the hypocotyl region of GFP:TUB6 Arabidopsis seedlings. MTs from (A) control seedlings, and seedlings subjected to 4°C for (B) 0.5 h, (C) 1 h, (D) 24 h, or (E) 1 week. Images are Z stacked images of cortical MTs at cell surface, representative of approximately 70 cells observed for each treatment. Arrows indicate tubulin globules. Scale bars = 10 μm.

cells contained a combination of thickly bundled MTs and short MT fragments and tubulin globules (Figure 2.3B).

The appearance of diffuse fluorescence surrounding existing MTs and bright fluorescent patches in vesicular structures and small granules in the cell cortex often attached to MTs (Figure 2.3 arrows) supports the observation of MT shortening in cold acclimated cells. Tubulin granules appear to be formed when free GFP labelled tubulin proteins become saturating following cortical MT disorganization (Ueda and Matsuyama 2000). Similar tubulin patches in vesicle-like structures and diffuse formations have been described following MTs disorganization resulting from osmotic stress (Blancaflor and Hasenstein 1995) and low temperature stress (Ahad et al. 2003). Diffuse patches of tubulin fluorescence have also been observed when actin filaments are disrupted (Ueda and Matsuyama 2000). It is possible that the observed disk-like tubulin structures are attributable to plasma membrane disruptions, because similar disk-like structures have been observed in membranes when they appear to fold into the extracellular space following low temperature stress and mechanical damage (Yamazaki et al. 2005).

Mid-length MTs in both disorganized and partially parallel arrays dominated 1 day acclimated plants (Figure 2.3D). Following acclimating treatments in excess of one day, the MTs appear to transition from cold induced disorganization and shortening to reformation and lengthening. As the period of acclimation increased MTs regained both length and parallel organization, until after approximately one wk at 4°C the cortical MT arrays are indistinguishable from those of untreated plants (Figure 2.3E).

Acclimating temperatures also affected the presence and organization of tubulin beneath the cortical arrays. Untreated cells usually contained only individual MTs and relatively thin MT bundles in transvacuolar strands and cortical MTs that remained attached to the plasma membrane and rarely separated from the outer edge of the cell cortex (Schulz 1988; Mandelkow and Mandelkow 1995; Figure 2.4A). After only short periods (30 min - 1 hr) at 4°C tubulin was seen at greater distances from the edge of the cell and occupied more cellular space than in untreated cells (Figure 2.4B). The number and thickness of MT bundles protruding into the cellular space and in cytoplasmic strands crossing the cell also increased in proportion to the increased time of exposure to acclimating temperatures, and many thick bundles of MTs transversed the lumen of long term acclimated plants (Figure 2.4C and D). Tubulin also was found in the nucleus of some acclimated plants (Figure 2.4B and C). Generally, short term

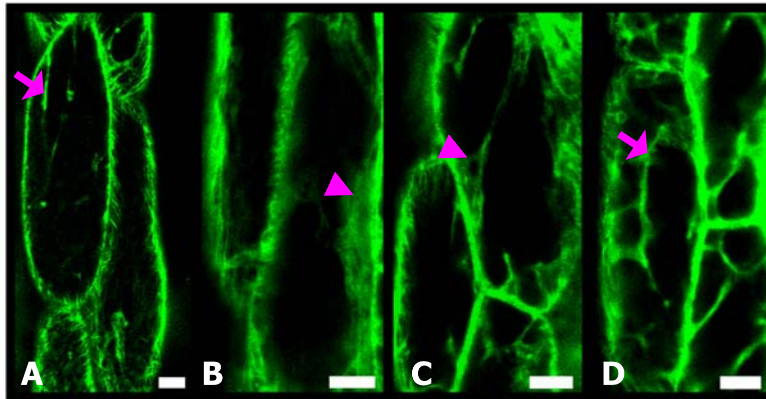

Figure 2.4. Confocal micrographs of tubulin accumulation beneath cortical MTs of GFP:TUB6 Arabidopsis seedlings. Tubulin fluorescence from cross-section of epidermal cells through the region of the nucleus from (A) untreated Arabidopsis seedlings and seedlings subject to 4°C for (B) 1 h, (C) 3 d, and (D) 1 week. Arrowheads indicate nuclei, arrows indicate interior cytoplasmic MT bundles. Scale bars = 10 µm.

acclimated plants only contained nuclear tubulin in a few cells, with the proportion of cells with intranuclear tubulin increasing with the duration of cold acclimation treatment.

2.3.3 Microtubule Disrupting Chemicals and Acclimation

2.3.3.1 Effect of microtubule disrupting chemicals on microtubule organization

WT and GFP:TUB6 plants were grown on low concentrations of MT disrupting chemicals to examine the effect of reduced MT assembly and disassembly on Arabidopsis growth orientation, MT organization and low temperature tolerance.

Roots of WT and GFP:TUB6 Arabidopsis seedlings grown vertically on ½ MS medium grew in a loosely waving helical arrangement skewed slightly to the left side of vertical hard agar plates (Figure 2.5D). Growth on 1 µM - 2 µM pronamide or 50 nM - 100 nM oryzalin substantially reduced the root waving and on occasion slightly stunted the root growth while the leftward growth angle was maintained (Figure 2.5E). The cotyledons and rosette leaves of some plants grown on 1 µM - 2 µM pronamide became slightly twisted in a clockwise manner (Figure 2.5B). The leaf phenotype of oryzalin treated seedlings did not visibly differ from control plants (Figure 2.5A). Treatment with 100 nM - 300 nM concentrations of taxol did not visibly affect root growth but promoted clockwise twisting of cotyledons and rosette leaves (Figure 2.5C).

MT arrays of plants treated with low concentrations of pronamide and oryzalin appeared more dense and organized than those of non-treated plants (Figure 2.6A - C). When grown on 300 nM taxol the plant MT arrays were more parallel with longer MTs than untreated MT arrays, and sometimes formed long swirling patterns across the cell (Figure 2.6D).

Concentrations of oryzalin and pronamide between 10 and 20 µM caused considerable visible MT disassembly and disorganization (Figure 2.6E and H). Arabidopsis plants subjected to 12- 24 h treatments on the chemicals contained cortical MT arrays with relatively short MTs and non-MT tubulin fluorescence primarily in the form of vesicular patches (Figure 2.6E and H). Intranuclear tubulin also accumulated following the chemically induced MT disassembly (Figure 2.6F and I) at a frequency similar to 2-4 day acclimated plants (Figure 2.4C). Contrary to low temperature affects however, no significant inward movement of tubulin or increase in MT bundles transversing the cell accompanied the chemical treatments. Long MT arrays reformed in plants returned to control medium for 12 - 24 h following a short chemical treatment (Figure 2.6G and J).

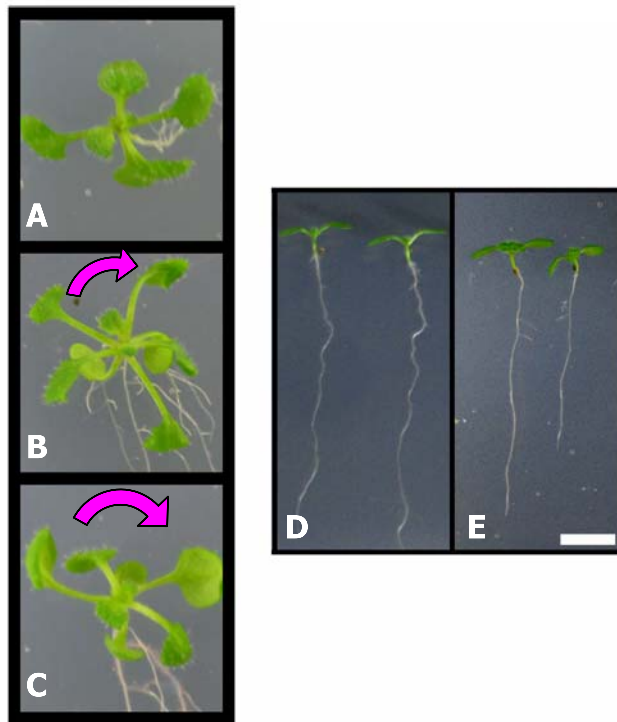

Figure 2.5. Effect of low concentrations of MT disrupting chemicals on seedling growth phenotype. Rosette leaf growth of seedlings grown for 10 days on (A) standard ½ MS medium, (B) MS medium containing 2 μM pronamide, or (C) MS medium with 300 nM taxol. Arrows indicate direction of leaf twisting. Root growth of seedlings grown for 7 days on (D) standard ½ MS medium or (E) MS medium containing 100 nM oryzalin. Scale bar = 5 mm.

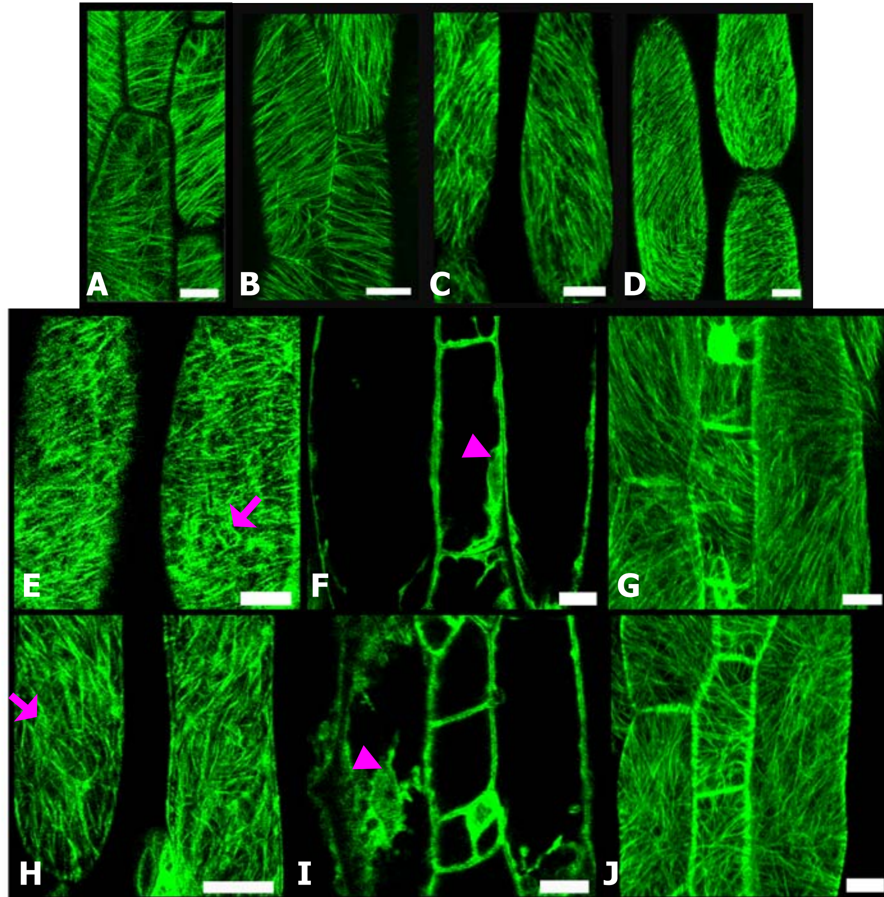

Figure 2.6. Confocal micrographs showing the effect of MT disrupting chemicals on MT organization in GFP:TUB6 Arabidopsis seedlings. (A) MTs of untreated control seedlings. (B-D) Seedlings with highly organized MTs after continuous treatment with (B) 0.5 μM oryzalin, (C) 3 μM pronamide, and (D) 3 μM taxol. (E-J) MT disassembly and reorganization induced by treatment with high concentrations of MT disrupting chemicals. MT fragmentation and disorganization following 12 h treatment with (E-F) 20 μM pronamide or (H-I) 10 μM oryzalin. MT reformation after 24 h recovery periods after 10 μM pronamide treatment or (J) 10 μM oryzalin treatment. Images F and I are cross-sections taken through epidermal cells at nuclear plane, remaining imges are Z stacked images of cortical MTs at cell surface. Arrowheads indicate nuclei, arrows indicate vesicular patches. Images are representative of approximately 40 cells observed for each treatment. Scale bars = 10 μm.

2.3.3.2 Effect of microtubule disrupting chemicals on freezing tolerance

The effect of MT disrupting chemicals on plant freezing tolerance was measured by electrolyte leakage freezing tests. The freezing tolerance of Arabidopsis seedlings grown on low concentrations of pronamide, oryzalin and taxol was compared with the freezing tolerance of untreated WT seedlings under both acclimating and non-acclimating conditions. There was no significant difference in freezing tolerance observed between any of the non-acclimated plants, nor between control and oryzalin-treated one wk acclimated plants. However, freezing tolerance of 1 wk acclimated pronamide- and taxol-treated plants was reduced significantly, from an LT_{50} of -7.5°C in WT to -5.8°C following pronamide treatment and -4.8°C after taxol treatment (Figure 2.7).

The freezing tolerance of plants treated for 24 h with 10 µM oryzalin both with and without a recovery period did not differ significantly from control plant freezing tolerance (Figure 2.8). Treatment with 20 µM pronamide for 24 h caused a slight decrease in freezing tolerance, but when this treatment was coupled with a 24 h post treatment recovery period without pronamide, freezing tolerance was increased by over 0.5°C (Figure 2.8). Other chemical concentrations and time period combinations were carried out, but no oryzalin treatment resulted in any increase in freezing tolerance, while 12 and 24 h pronamide treatments had the most significant effect on freezing tolerance.

2.4 Discussion

2.4.1 Low Temperature Causes Different Levels of Microtubule Disassembly

In this study, MTs were consistently observed undergoing disassembly in Arabidopsis plants exposed to low temperatures. The degree of MT disassembly was dependent on the duration and severity of the low temperature treatment.

MTs were either entirely or almost entirely disassembled when exposed to severe temperature stress (e.g. long term (24 h) at 0°C or short term (4 h) at -6°C), and did not resassemble while exposed to these temperatures. Acclimation at 4°C caused MTs to initially partially disassemble and disorganize during the first day of low temperature exposure before gradually reforming into arrays that resembled those of control cells after one week of treatment. The MT disassembly that occured during cold acclimation was much less severe than that caused by cold shock. Although different levels of MT disassembly may be directly related to the

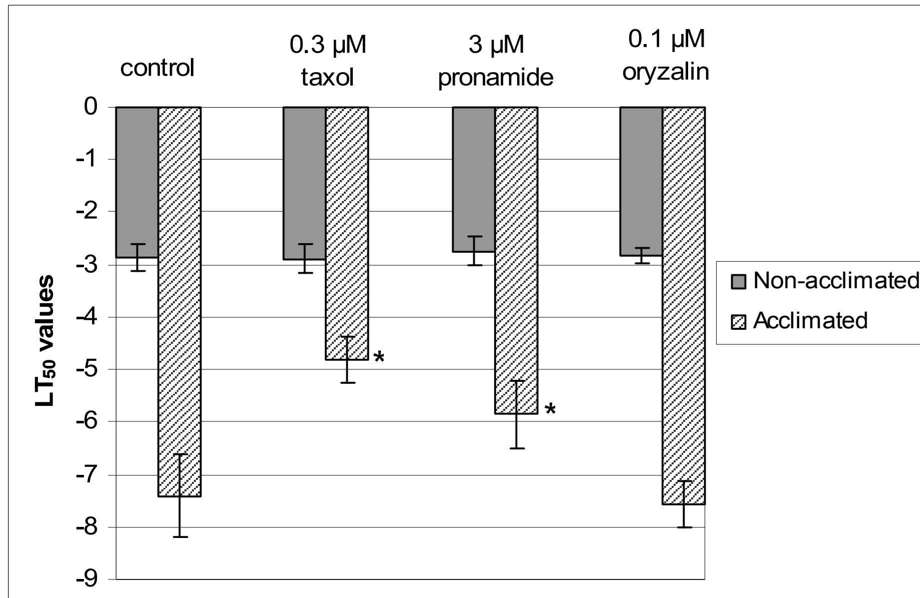

Figure 2.7. Freezing tolerance of WT Arabidopsis grown on media containing low concentrations of MT disrupting chemicals (0.3 μM taxol, 3 μM pronamide, or 0.1 μM oryzalin). Freezing tolerance was determined by electrolyte leakage tests measured before and after 1 week of cold acclimation at 4°C. Error bars represent standard errors of means (n=3). * Indicates significant difference from control ($p \leq 0.05$), determined by Student's t-test.

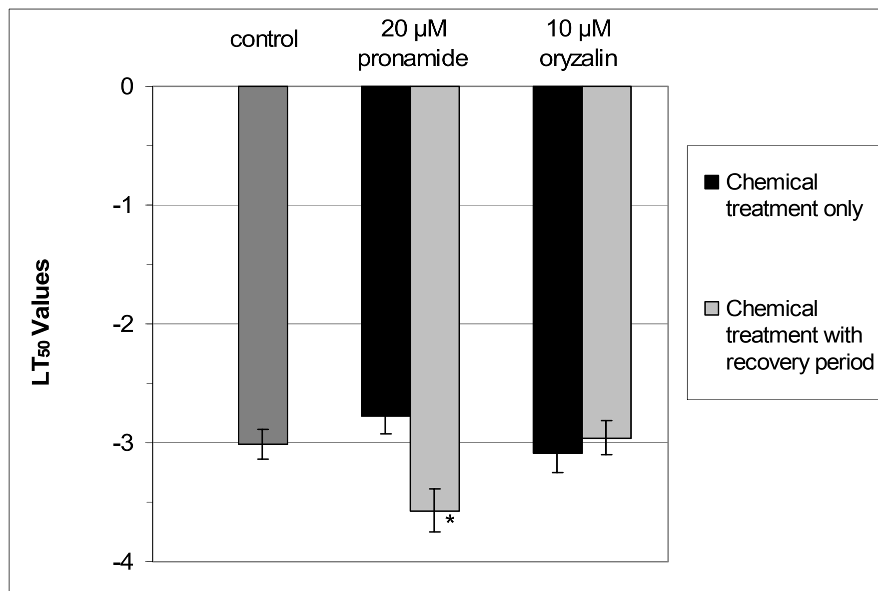

Figure 2.8. Freezing tolerance of WT Arabidopsis grown on control MS media transferred to 20 μM pronamide or 10 μM oryzalin medium for 24 h. Freezing tolerance was determined by electrolyte leakage tests measured immediately following chemical treatment (chemical treatment only) or 24 h after treatment (chemical treatment with recovery period). Error bars represent standard errors of means (n=3). * Indicates significant difference from control ($p \leq 0.05$), determined by Student's t-test.

severity of the stress, the ability of MTs to repolymerise and reform into long and highly organized MTs while in the presence of a low temperature stress indicates that there are important differences between 0°C and 4°C temperature treatments on MTs. The persistence of some MTs throughout the cold acclimation process, and the ability of MTs to polymerise while being exposed to low temperature suggest that the condition or signal that induces MT depolymerization is removed following a short period at chilling temperatures.

MT disassembly following various low temperature exposures could occur by different means. Low temperature causes both in vitro and in vivo MT disassembly by internal MT severing and the loss of MT dimers at the MT ends (Mandelkow and Mandelkow 1995). However, MT disassembly induced by cold shock is probably a result of direct depolymerization by the cold. MTs are also known to depolymerise in response to signals other than temperature including plasma membrane rigidification and the binding of Ca^{2+} ions; signals known to occur during the early cold acclimation process (Bokros et al. 1996; Breviario and Nick 2000; Sangwan et al. 2001). Cold acclimation temperatures may directly promote MT disassembly, or any individual signal or combination of other signals may be responsible for causing MTs to undergo the incomplete and transient MT disassembly that occurs during cold acclimation.

Differences in disassembly and polymerization of MTs following the initial depolymerization may be related to changes in the MT itself. Inherently cold stable MTs have been described in plant and animal species that survive and grow in low temperature environments, and subsets of MTs resistant to temperature-induced depolymerization have been described in Arabidopsis (Smertenko et al. 2004; Muller et al. 2007). The long MTs observed in cells containing otherwise short and disassembled MTs, and the continued presence of relatively long and parallel MTs in some cold shocked cells supports the existence of a population of cold stable MTs in non-acclimated Arabidopsis. The partial MT disassembly followed by MT polymerization that occurs during cold acclimation may represent the depolymerization of MTs containing cold sensitive tubulin proteins, followed by the repolymerization of MTs with tubulin proteins that are less sensitive to cold-induced destabilization. Cold acclimation may give the plant an opportunity to remove cold sensitive tubulin proteins and make MTs uniformly cold tolerant. The binding of different populations of MAPs, different tubulin PTMs, and/or changes to tubulin gene expression may be responsible for the tubulin/MT cold stability (Nick 2000).

2.4.2 Microtubule Involvement in Cold Acclimation

In order to determine if MT reorganization that occurs in cold acclimated Arabidopsis plants is important to the cold acclimation process or if it is occurring merely as a consequence of low temperature stress, MT disrupting chemicals were used to manipulate the ability of MTs to polymerise and depolymerise.

Some organisms contain MTs with reduced dynamics and slow polymerization rates that are inherently cold stable, and the reduced MT dynamics were proposed to increase cold-tolerance (Detrich et al. 2000). The importance of MT dynamics to cold tolerance was examined using treatments with low concentrations of pronamide, oryzalin and taxol, which are known to reduce MT dynamics (Derry et al. 1995; Nakamura et al. 2004). The reduction of MT dynamics was demonstrated by the slight twisting phenotype and highly organized patterns of thick MT arrays observed in this study (Figure 2.5 and 2.6A-C) that correspond to reports in the literature describing the effect of reduced MT growth and shrinkage rates (Nakamura et al. 2004; Ishida et al. 2007). Long-term treatment with low concentrations of these chemicals did not affect the cold tolerance of non-acclimated plants; plants survived to approximately -3°C whether or not they were treated with MT disrupting chemicals (Figure 2.7). Although slow moving MTs may still be more cold stable than dynamic MTs, in Arabidopsis their presence did not affect plant cold tolerance.

When plants were treated with pronamide or taxol during the acclimation treatment, their ability to reach their maximum level of freezing tolerance was inhibited, supporting the belief that MT dynamics are required for efficient/effective cold acclimation (Kerr and Carter 1990; Bartolo and Carter 1991; Örvar et al., 2000). However, oryzalin treatment did not significantly affect the ability to acclimate, and plants were still capable of acclimating to a relatively high level when MT dynamics were suppressed with pronamide (Figure 2.7). Although MT dynamics may be a part of cold acclimation, rapid depolymerization or repolymerization of MTs is not an absolute requirement for cold acclimation in Arabidopsis.

The three chemicals used in this experiment decrease MT dynamics by different mechanisms. Pronamide binds to β-tubulin to interfere with MT polymerization. When present in low concentrations it reduces overall MT dynamics while in high concentrations it promotes overall MT depolymerization (Akashi et al. 1988; Young and Lewandowski 2000). Oryzalin primarily binds to free α-tubulin to destabilize the binding of lateral protofilaments (Hugdahl and

Morejohn 1993). Oryzalin can also bind to polymerised tubulin to cause MT destabilization, but this effect is less pronounced than its effect on unpolymerised tubulin (Xiao et al. 2006). As well as its primary action in preventing MT depolymerization, taxol also bundles MTs when present in low concentrations (Elie-Caille et al. 2007). The ability of each of these chemicals to prevent MT depolymerization is reflected in their inhibitory effects on plant acclimation. This indicates that low temperature induced transient MT disassembly may still be involved in the early low temperature signalling pathway.

The effect these chemicals have on MAP binding is likely different for each chemical. Taxol causes the most dramatic changes to MT structure; it causes MT bundling and binds continuously to the MT afecting MAP binding to MTs (Chu et al. 1993; Rhee et al. 2003). The presence of taxol had the most severe effect on the ability of plants to acclimate; therefore the differences between taxol and oryzalin stabilized MTs could be good indicators of the MT characteristics important for cold acclimation.

It is possible that the changes in MT polymerization/depolymerization that occured during cold acclimation were directly related to changes in isoform composition of the MTs. The tubulin protein C-terminus, particularly the C-terminal end of β-tubulins, appears to be the target of low temperatures. β-tubulin proteins with long C-terminal ends are cold sensitive while tubulin proteins with short C-termini are more tolerant to low temperatures (Bokros et al. 1996; Redeker et al. 2004). Genes encoding tubulin proteins with long C-termini have low expression during low temperature exposure while cold exposure causes genes coding for tubulin proteins with short C-termini to be upregulated (Chu et al. 1993). Possibly the transient partial MT disassembly that accompanies cold acclimation occurs to remove cold sensitive tubulin proteins from MTs and replace them with cold tolerant tubulin proteins. Tubulin gene expression and protein degradation are regulated by complex mechanisms: excess tubulin proteins bind to their encoding mRNA to prevent the production of additional tubulin proteins of the same form and unpolymerized tubulin proteins that are properly sequestered and folded are degraded (Giani et al. 2002). The abundance of unpolymerized, cold-sensitive tubulin dimers would account for an altered level of gene expression and transient MT disassembly.

The presence of pronamide and oryzalin may similarly affect the population of tubulin isoforms being incorporated into the MTs. When pronamide and oryzalin are present in low concentrations MTs are able to slowly polymerise, and the tubulin isoforms that will incorporate

into the MT will be isoforms with the most resistance to chemically induced depolymerization. In some organisms, a single amino acid change contributes to both increased cold hardiness and resistance to MT disrupting benzimidazole chemicals (Redeker et al. 2004; Robinson et al. 2004). Although it is possible that tubulin proteins with cross resistance to cold and chemically induced depolymerization exist in Arabidopsis, this is unlikely because prolonged exposure to low concentrations of chemicals had no effect on freezing tolerance of non-acclimated Arabidopsis plants (Figure 2.7).

In order to determine if MT disassembly alone (in the absence of a low temperature signal) could induce changes in freezing tolerance cortical MT rearrangements observed in GFP:TUB6 plants (Figure 2.3 and 2.4) during the cold acclimation process were mimiked using the microtubule disrupting chemicals, pronamide and oryzalin. These treatments (when followed by a MT recovery period) were capable of causing similar visible changes in cortical MT organization to those observed during the early (Figure 2.6E, F, H and I) and later stages of acclimation (Figure 2.6G and J).

When the initial stages of low temperature treatment were mimicked by treatment with high concentrations of pronamide there were slight decreases in plant freezing tolerance, while oryzalin-induced MT disassembly had no effect (Figure 2.8). Similar to results obtained in previous studies (Örvar et al. 2000) when MTs were disassembled, plants did not have significant cold tolerance because intact MTs are required for plant growth and functioning.

When pronamide-induced MT disassembly was combined with a MT recovery period to better mimic the changes that occur during cold acclimation, plants showed increased freezing tolerance of nearly 20% over controls and nearly 30% over pronamide-treated plants without the recovery period (Figure 2.8). However, again freezing tolerance was not significantly changed by a treatment with oryzalin followed by the recovery period (Figure 2.8). The pronamide induced increases were significant, but the inability of oryzalin to induce similar changes and the increase of only -0.5°C following pronamide treatment compared to increases of >-4°C caused by cold acclimation suggest that there are elements of the cold acclimation process that can not be induced by simple manipulation of MTs.

The inability of chemically induced transient MT depolymerization to induce cold acclimation and large increases in freezing tolerance may be related to the inability of these chemicals to induce required changes to isoform composition, protein PTMs and MAP binding.

Chemical treatments are capable of inducing MT reorganization, but do not necessarily induce similar changes to the MAP population and PTMs that occur during cold acclimation. It is also possible that residual chemical remained within the plant during the freeze test, making MTs more prone to disassembly.

Disruption of MTs by chemicals and low temperatures affects interactions between the MTs and membranes. Previous studies have described the relationship between the plasma membrane and MTs and have hypothesized that low temperatures disrupt these interactions (Akashi and Shibaoka 1991). When exposed to low temperature stress membranes change their properties and become very rigid. Possibly the requirement of MT disassembly is related to a need for MTs to dissociate from the plasma membrane, thereby preventing MT breakage and a spread of structural damage to the rest of the cell. The effects of oryzalin and taxol on the ability of plants to cold acclimate support this idea. MTs treated with oryzalin can be severed throughout their length, and this severing may help reduce MT membrane interactions and reduce structural damage. Taxol affects MT structure and MAP binding more than MT destabilizers and therefore it is more likely to affect interactions between MTs and cellular elements such as membranes. This effect on membrane interactions may be responsible for the larger effect taxol had on cold acclimation.

In this study both low temperature and chemically induced MT disassembly promoted intranuclear tubulin accumulation. Interphase nuclei normally exclude tubulin from the nucleus and tubulin does not appear to contain import signals (Schwarzerová et al. 2006). However, the significant levels of intranuclear tubulin occur against the concentration gradient and nuclear pores are smaller than tubulin dimers, therefore simple diffusion across damaged membranes cannot explain the presence of intranuclear tubulin.

Although animal and plant cells undergo open mitosis, some components of the spindle regulation machinery (e.g. γ-tubulin) are imported into the nucleus before mitosis, and tubulin must be removed from the nucleus when daughter nuclei form new nuclear membranes (Schwarzerová et al. 2006). It is unclear precisely how the tubulin is excluded from the nucleus but five putative NES exist in α- and β- tubulins. It has been suggested that tubulin enters the nucleus following low temperature stress because the low temperatures either damage the nuclear envelope or overload the nuclear pore complexes (NPCs) to cause the normally active tubulin export mechanisms to fail (Schwarzerová et al. 2006). Because chemicals also cause

intranuclear tubulin accumulation, it is probable that the machinery that removes tubulin at the end of mitosis acts continuously during interphase, and these export mechanisms fail when the cell contains high levels of free tubulin.

The presence of intranuclear tubulin does not necessarily require membrane or machinery damage. NPCs and nucleocytoplasmic trafficking are important to many functional regulation pathways, including cold tolerance, disease resistance, hormone signalling, and development (Dong et al. 2006; Xu and Meier 2008). Large proteins and complexes (e.g. ribosome subunits) that are much larger than nuclear pores are regularly transported across the NPC (Schäfer et al. 2003). The presence of tubulin and MAPs within the nucleus is normally carefully regulated and the association of tubulin with chromatin is essential for cell cycle regulation. The consequence of intranuclear tubulin on gene expression and cell cycle regulation during low temperature stress has yet to be examined, but it is possible that nuclear tubulin affects cold induced gene expression (Schwarzerová et al. 2006).

This study examined the relationship between low temperature stress, cold acclimation and microtubule organization. The effects of low temperatures on MT organization were observed and the potential importance of this reorganization was examined. This study confirmed that low temperatures affect MT organization and that MTs are involved in plant cold acclimation, and was the first to visualize MTs in a live plant system. This was the first report of intranuclear tubulin accumulation occurring as a result of chemically induced MT disassembly, and challenged the idea that MT disassembly is responsible for triggering the cold acclimation pathway. Instead, this study indicates that a variety of changes to tubulin and MTs occur during low temperature exposure to facilitate the complex role MTs play in the acquisition of freezing tolerance in Arabidopsis.

CHAPTER 3
PROTEOMIC STUDY OF COLD INDUCED CHANGES IN ARABIDOPSIS TUBULIN BINDING PROTEINS

3.1 Introduction

MTs are essential cytoskeletal elements involved in mitosis, cytokinesis, cell polarity, intracellular trafficking and other fundamental processes. The various functions of MTs are dependent on MT growth and shrinkage dynamics and the distribution and arrangement of MTs throughout the cell. MAPs are responsible for determining MT structure, dynamics, organization and interaction with other cellular elements, and thus control MT functioning (Wasteneys and Galway 2003; Sedbrook 2004). Recent progress in the identification and characterization of new MAPs has been incremental (Hamada 2007; Kaloriti et al. 2007).

Some proteins bind to MTs without affecting MT activity. The association of these proteins with MTs may be important in a variety of ways, such as regulating protein activity and localization, or interaction with other proteins and cellular elements (Chuong et al. 2004). The relatively recent discovery that MTs are involved in modulating plant responses to changes in their environment most probably involves the activity of proteins that associate with but do not regulate MTs, such as signalling proteins that interact transiently with MTs. Unfortunately the association of such proteins with MTs in plants is currently poorly understood.

The ability of plants to respond to their external environment requires the activity of sensors and receptors at the cell surface. The identity of the cellular element(s) involved in sensing low temperatures in plants is not known. MTs are known to respond to various external stimuli, and disassemble very rapidly in response to low temperatures. Preventing MT disassembly impedes the ability of plants to tolerate low temperature, leading some to propose that MTs are the low temperature sensors in plants (Abdrakhamanova et al. 2003). However, MT disassembly has very different effects on low temperature tolerance when induced by chemicals in different plant species. For example, pronamide induced MT disassembly followed by MT reassembly caused significant increases in freezing tolerance of winter wheat roots (Abdrakhamanova et al. 2003) while the same process increased freezing tolerance only slightly

in Arabidopsis plants (Chapter 2). When antimicrotubular drugs were applied to chilling sensitive plants during chilling, however, it increased damage (Rikin et al. 1980). Treatment with low concentrations of taxol, pronamide and oryzalin all reduce MT dynamics to very similar levels, but affect a plants' ability to cold acclimate to very different extents (Chapter 2). Because these chemicals affect the interactions between MTs and MAPs differently, and because MAPs control MT activity and therefore many other cellular functions, MAPs are therefore better candidate low temperature sensors and signal transmitters than other MT components.

Changes in MAPs in control vs. low temperature exposed plants may occur for a variety of reasons that would affect the low temperature response of a plant in a range of ways. A decrease or increase in binding of MT stabilizing or destabilizing MAPs could facilitate MT disassembly. Changes in MAP binding may be important for MT repolymerisation during the cold acclimation process. Altered gene/protein expression or altered protein-MT binding affinity could cause the different MT-MAP binding. Additionally, MAPs have functions beyond MT binding, such as triggering kinase cascades and other responses related to different biochemical pathways, therefore they may participate in the cold signalling process by both affecting MT stability and participating in signalling cascades important to the plant's cold response (Ahad et al. 2003; Chuong et al. 2004; Nakamura et al. 2004). Low temperatures could affect MAPs that mediate the relationship between MTs and the plasma membrane and extracellular matrix, thus influencing stress sensing and signalling. Additionally, the association of proteins with MTs could control the localization and/or biological activity of the protein and various aspects of the stress response.

MAPs with a variety of cellular functions have been identified using different techniques. MAPs involved in controlling MT functions such as cell division and morphology have been identified by screening mutant populations for plants with altered morphology and flawed cell division (Smith et al. 1996; Furutani et al. 2000). In silico searches for orthologues to animal MAPs have identified proteins involved in controlling MT structures and dynamic instability that are conserved among these organisms (Whittington et al. 2001). The identification of proteins that bind to MTs regardless of their impact on MT activity can be identified by tubulin affinity chromatography (Durso and Cyr 1994; Chuong et al. 2002; Chuong et al. 2004). MAPs involved in stress signalling will probably interact transiently with the cytoskeleton rather than directly

binding to controlling MT activity. Tubulin affinity chromatography is therefore the most useful technique to identify MAPs involved in stress responses.

Chuong et al. (2004) used tubulin affinity chromatography to identify many tubulin/MT interacting proteins, some (e.g. signalling proteins) with the potential to be involved in the low temperature stress response. However, this and other studies investigating MAPs used cell suspension cultures or protoplasts which do not necessarily represent the structure and interactions that occur in cells within the whole plant environment (Zilkah and Gressel 1977). Cell suspension cultures have both advantages and disadvantages over whole plant studies. They are relatively easy to generate and provide an easily manipulated homogeneous tissue that simplifies the proteome and the examination of cellular responses. However, it is also impossible for cell cultures to properly demonstrate whole plant responses, and tissue from whole plants and cell suspension cultures have different protein and transcript responses to low temperature stress (Chen et al. 1983).

In this study, tubulin binding proteins from Arabidopsis whole plant protein extracts were identified. Tissue from whole plants was chosen over cell suspension cultures to better demonstrate actual protein-MT interactions occurring in nature. Combining results of this study with the results of Chuong et al. (2004) will provide a more comprehensive view of proteins that bind tubulin in Arabidopsis and will recognize differences between proteins identified from whole plant versus cell suspension cultures.

Protein samples from both untreated and short term cold-acclimated Arabidopsis plants were examined in order to identify tubulin binding proteins potentially involved in the cold acclimation process and the acquisition of freezing tolerance. Low temperature exposure has been shown to affect MT binding of some MAPs, but this has not been examined in detail. No large scale study of the effect of different conditions on protein tubulin binding has been performed. The early stages of cold acclimation cause the most dramatic changes in MT organization (Chapter 2), and therefore should present a MAP population with significant variation from control. Identifying tubulin binding proteins and their changing affinity for tubulin during the early stages of cold acclimation will help us understand why MTs change their organization during cold acclimation, and may indicate how MTs are involved in the cold acclimation process.

3.2 Materials and Methods

3.2.1 Plant Materials and Low Temperature Treatments

Arabidopsis (ecotype Columbia) seeds were sown in Coco soil-less mix and grown at 22°C with a 16 h photoperiod, 200 µmol/$m^{-2}s^{-1}$ light intensity. After four weeks of growth (before bolting, approximately 12 leaves), some plants were transferred to a cold chamber (4°C, 150 µmol/ $m^{-2}s^{-1}$ light intensity) for 1 hour (h) or 1 day (d) low temperature treated (LT) treatment. Leaf samples were harvested and immediately frozen in liquid nitrogen and stored at -80°C until protein extraction.

3.2.2 Preparation of Protein Extract

The protein extract was prepared essentially as described previously (Chuong et al. 2002). Approximately 50 g of aerial tissue from 1 h and 1 d LT and untreated Arabidopsis plants was ground to a fine powder in liquid nitrogen and 30 ml of cold extraction buffer (100 mM HEPES adjusted to pH 7.5 with 3 mM KOH, 5 mM $MgSO_4$, 5 mM EGTA) containing complete protease inhibitor cocktail tablets (Roche Applied Science). The crude protein extract was filtered through one layer of Miracloth, centrifuged for 0.5 h at 50 000 x g, and the supernatant re-centrifuged at 100 000 x g at 4°C to remove cellular debris and insoluble proteins. Protein concentrations were determined by the Bradford method (Bradford 1976) using bovine serum albumin (BSA) as standard. Plant material

3.2.3 Preparation of Protein Coupled Columns

Bovine brain tubulin affinity columns were prepared essentially as described previously (Chuong et al. 2002) at 4°C. CNBR-activated CH-Sepharose 4B (Amersham Biosciences) was swollen and washed on a sintered glass filter with ice cold 1 mM HCl for 0.25 h, followed by equilibration of the matrix in column wash buffer (100 mM HEPES adjusted to pH 7.5 with 3 mM KOH, 5 mM $MgSO_4$, 5 mM EGTA, 50 mM KCl). Two ml of swollen Sepharose and 10 mg (>99%) pure bovine brain tubulin (Cytoskeleton, Inc) were coupled overnight at 4°C while rotating end-over-end. The mixture was gently loaded onto an empty PD-10 column (Amersham Biosciences) and washed with approximately 40 ml coupling buffer to remove unbound tubulin. The remaining active groups were blocked by washing the column with 0.1 M Tris-HCl buffer, pH 8.0, and the column was incubated in this buffer overnight. The column medium was washed with three cycles of alternating high and low pH buffers (high pH buffer: 0.1 M Tris-HCl (pH 8.3) containing 0.5 M NaCl; low pH buffer: 0.1 M sodium acetate (pH 4.0) containing 0.5 M

NaCl). Columns were prepared for sample loading by washing with elution buffer, to ensure no unbound proteins remain within the columns, followed by equilibration in protein extraction buffer. BSA control columns were also prepared with an equivalent amount of protein and the same procedure as the tubulin column.

3.2.4 Tubulin Affinity Chromatography

Seventy-five mg of purified Arabidopsis proteins were applied to the bovine brain tubulin columns by drip loading at an approximate rate of 4 mg per h. Unbound proteins were removed by washing the columns with 50 column volumes (100 ml) wash buffer containing protease inhibitor tablets. Proteins that bound to the affinity column were eluted from the column using wash buffer containing a total of 500 mM KCl, and eluate was collected in 18 x 0.5 ml fractions. Column loading and elution were performed at 4°C. For each replicate, control columns were prepared and run alongside treatment columns using plant material grown concurrently.

3.2.5 Gel Electrophoresis - SDS-PAGE

Eluates from the tubulin-Sepharose columns were loaded on a one dimension (1D) sodium dodecyl sulfate (SDS) polyacrylamide gel consisting of a 1.5 x 15 x 3-cm 5% stacking gel over a 12% (1.5 x 15 x 10-cm) resolving gel. After 1D SDS polyacrylamide gel electrophoresis (PAGE) separation proteins were visualized by Bio-Safe Coomassie G250 stain (Bio-Rad) or by silver staining (Silver Stain Plus, Bio-Rad).

3.2.6 Mass Spectrometry

Tubulin binding proteins from control, 1 h and 1 d LT plants were loaded onto a 1D SDS-PAGE gel and resolved within a 5 mm section of the resolving gel in order to maximize the amount of protein in a small volume of gel. Gel segments containing each protein sample were divided into two segments to improve protein isolation during mass spectrometry analysis. Liquid chromatography- tandem mass spectrometry (LC-MS/MS) analysis was performed on an ESI-Quad-TOF QStar XL or an Agilent MSD Ion Trap XCT interfaced with an Agilent 1100 series Nano LC system at the Southern Alberta Mass Spectrometry (SAMS) Centre for Proteomics. MS/MS spectra were processed and searched against the Mass Spectrometry protein sequence DataBase (MSDB) and the TAIR database using the MASCOT algorithm (Perkins et al. 1999; Rhee et al. 2003). Only significant protein hits (as defined by the MASCOT probability analysis, identified with a confidence of $p<0.05$) in at least two of the three independent biological and technical replicates were included in the results. Functional categorization and

subcellular localization of identified proteins was performed using the TAIR database (www.arabidopsis.org; Rhee el al. 2003).

3.3 Results

3.3.1 Purification of Tubulin Binding Proteins

Tubulin affinity chromatography was used to separate tubulin-binding proteins from plant protein extracts. A comparative analysis of protein binding to bovine and plant microtubule- and tubulin-affinity chromatography columns indicated that the protein fractions isolated by each of the column types are very similar, differing primarily with respect to protein abundance (Durso and Cyr 1994; Chuong et al. 2002; Chuong et al. 2004). Although there are likely to be some differences in the binding of proteins to bovine brain tubulin compared to tubulin and MTs in plants, the difficulties in obtaining pure plant tubulin in the required quantities to perform this study dictated the use of bovine brain tubulin rather than plant tubulin to isolate plant MT and tubulin binding proteins.

BSA shares many features with tubulin including a similar isoelectric point and molecular mass, and were therefore used as a control to identify any non-specific protein binding. A small number of proteins were observed in the BSA column eluate (Figure 3.1B) while numerous Arabidopsis proteins were eluted from the tubulin column (Figure 3.1A). Analysis of the proteins present in the BSA elutions found two potential contaminant proteins; a histone H2 and a lipase/acylhydrolase, which likely interact with the columns by non-specific ionic interactions and are thus considered contaminants in all tubulin binding columns and were not included in the list of Arabidopsis tubulin binding proteins. Tubulin affinity chromatography will also isolate proteins that indirectly associate with MTs by their binding to tubulin binding proteins.

Examination of silver stained 1D-SDS PAGE gels of tubulin binding protein fractions from different replicates and temperature treatments revealed consistent patterns of protein bands between replicates, with some variation related to band intensity. Variation in band intensity and location in different sample types was observed (Figure 3.1). Because of the limited resolution of 1D-SDS PAGE gels, it is probable that other protein differences were present but not detectable in the gels.

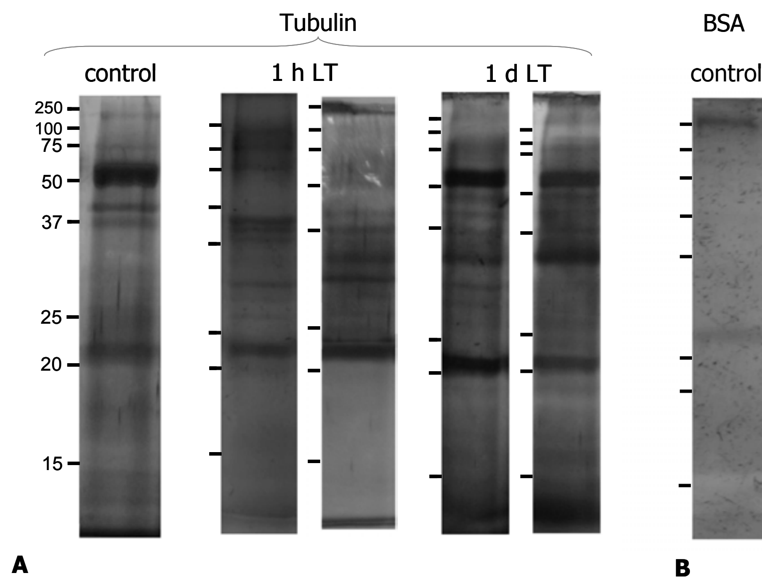

Figure 3.1. Silver stained 1D SDS-PAGE gels of Arabidopsis proteins that bound to tubulin affinity columns. Variation in protein binding from different treatment types and replicates were observed in (A) control Arabidopsis tissue and 1 h and 1 d low temperature treated tissue (two replicates each). (B) Gel segment of control BSA column showing nonspecific protein binding. The molecular mass standards are indicated to the left of each gel, with corresponding kDa values at the left of the image.

3.3.2 Identification of Tubulin Binding Proteins by LC-MS/MS

In total, 96 proteins from tubulin-binding protein fractions of control and 1 h and 1 d 4°C treated Arabidopsis plants were identified in at least two replicate samples by LC-MS/MS (Table 3.1). There were 47 tubulin binding proteins identified in control plants, 81 from 1 d low temperature treated plants and 49 from 1 h low temperature treated plants. As shown in Figure 3.2 the majority of proteins identified in the analysis were present in multiple sample types. However, five proteins were identified only in the control, and 31 identified only in low temperature treated plants (Figure 3.2), representing 24 and 7 proteins unique to the 1 d and 1 h LT plants, respectively.

Despite similar protein banding patterns there was some variation in the number of proteins identified in the different replicates. Some of the differences in numbers may reflect the caveats of 1D-PAGE LC-MS/MS protein identification. Although 1D-PAGE LC-MS/MS is less biased towards high abundance proteins than 2D-PAGE, when a dominant protein or group of proteins is present in a sample, lower abundance peptides can be overshadowed when they share similar mass to charge (m/z) properties with the more abundant peptides. Protein identification could have been limited in this way here. Alternatively when proteins with high affinity binding to tubulin are present in high levels they are able to out-compete proteins with lower affinity to tubulin, resulting in similar limited protein identification.

3.3.2.1 Predicted cellular distribution

The relative distribution of identified proteins (Table 3.1) believed to be associated with various cell components is presented in Figure 3.3. Membrane proteins (e.g. endomembrane proteins) were the largest group (33%), followed by cell wall proteins (12%) and proteins in the cytoplasm (11%). Proteins annotated as nuclear, plastid and mitochondria-localized made up 3, 6 and 4% of the tubulin binding proteins, respectively.

The protein localization results are based on data currently present in databases obtained through experimental methods, from inferences based on sequence or structural similarity, and from computational prediction analysis. Unfortunately the annotations have not been tested for accuracy, and unaccountable errors may be present in this data.

The majority of identified proteins annotated as 'other membrane' proteins were endomembrane proteins with annotation inferred from N-terminal amino acid sequence analysis prediction using TargetP computational analysis software (Emanuelsson et al. 2000).

Table 3.1 Arabidopsis tubulin binding proteins identified by LC-MS/MS.

Proposed function	Spot #	AGI gene locus	Fraction found	Gene index number	Name	Calculated molecular weight(Da)/pI
nucleic acid binding	1	AT5G51280	C *	15241415	DEAD/DEAH-box RNA/DNA helicase, putative	144480/8.07
	2	AT2G21660	1 d	21553354	glycine-rich RNA binding protein 7; ATGRP7	16934/5.85
	3	AT5G47210	C; 1 h *	21553991	nuclear RNA binding protein A-like protein, putative	38000/9.35
	4	AT4G16830	C *	15235894	nuclear RNA-binding protein	37468/8.63
	5	AT5G07030	C; 1 h; 1 d *	9759559	nucleoid DNA-binding-like protein	47687/9.30
	6	AT3G07590	C; 1 d *	15231485	small nuclear ribonucleoprotein D1, putative	12592/11.22
	7	AT3G59980	1 d *	15232216	tRNA-binding region domain-containing protein	29898/7.71
	8	AT4G13780	1 h *	15236350	methionyl-tRNA synthetase; tRNA-methionine ligase	89854/7.00
protein metabolism	9	AT3G54400	C; 1 h; 1 d	15232503	aspartyl protease family protein; pepsin A	45476/9.30
	10	AT5G60390	C; 1 h; 1 d *	295789	elongation factor 1-alpha	49503/9.64
	11	AT2G33530	1 d *	2459435	serine carboxypeptidase II, putative	51514/8.66
	12	AT4G10540	1 h	4115919	similar to the subtilase family of serine proteases	83519/6.47
	13	AT5G51750	C; 1 h	18423316	subtilase family protein	84951/9.78
	14	AT1G71950	C; 1 d	18409953	subtilase; identical protein binding	14900/5.76
	15	AT2G04160	C; 1 h	3695019	subtilisin-like protease	82874/9.87
	16	AT4G34980	C; 1 d	18418552	subtilisin-like serine protease 2	81047/9.7
	17	AT3G04820	C; 1 d	145338120	pseudouridylate synthase	79402/5.96
cell responses	18	AT4G35090	1 d	16215	catalase 2	57192/6.75
	19	AT1G20620	1 d	2347178	catalase 3	57060/8.31
	20	AT2G34810	1 h	15226834	FAD-binding domain-containing protein	61308/10.11
	21	AT1G75750	1 d	887939	GA-responsive GAST1 protein homolog	11395/9.40
	22	AT5G58390	1 d	15237187	peroxidase, putative	35142/9.83
	23	AT4G37930	1 d *	15235745	serine hydroxymethyltransferase 1; SHM1	57535/8.13
	24	AT5G09440	C; 1 h; 1 d	15242420	phosphate-responsive protein, putative	29474/9.86
	25	AT5G64260	C; 1 h; 1 d *	15237656	phosphate-responsive protein, putative	32675/9.07
	26	AT1G23010	C; 1 h; 1 d	12323429	spore coat protein-like protein; multi-copper oxidase	66166/9.56
	27	AT1G18250	1 d	8671776	thaumatin-like protein T10O22.21	25978/8.53

carbohydrate metabolism	28	AT3G18080	C; 1 h; 1 d	15229584	glycosyl hydrolase family 1 protein	58984/9.82
	29	AT5G36890	C; 1 h; 1 d	22327412	glycosyl hydrolase family 1 protein	56077/5.35
	30	AT3G18070	1 d	9294062	glycosyl hydrolase family 1 protein; beta-glucosidase	56574/6.18
	31	AT5G25980	C; 1 h; 1 d	5107821	glycosyl hydrolase family 1 protein; thioglucosidase	62733/7.49
	32	AT3G47010	C; 1 h; 1 d	15232707	glycosyl hydrolase family 3 protein	67252/5.08
	33	AT5G20950	C; 1 h; 1 d	22326920	glycosyl hydrolase family 3 protein	33950/9.02
	34	AT1G02640	1 d	110740481	glycosyl hydrolase family 3 protein; beta-xylosidase	82986/8.42
	35	AT5G49360	C; 1 h; 1 d *	15239867	glycosyl hydrolase family 3 protein; beta-xylosidase 1	83524/8.61
	36	AT1G78060	C; 1 h; 1 d	6573772	glycosyl hydrolase family 3 protein; F28K19.27	83893/8.12
	37	AT5G13870	C; 1 h; 1 d	15240733	glycosyl hydrolase family 16 protein; endoxyloglucan transferase A4	33950/9.00
	38	AT2G06850	C; 1 h; 1 d *	15225605	glycosyl hydrolase family 16 protein; endoxyloglucan transferase A1	34291/9.20
	39	AT3G55430	C; 1 h; 1 d *	15028379	glycosyl hydrolase family 17 protein; beta-1,3-glucanase, putative	48443/9.74
	40	AT4G39640	C; 1 h; 1 d	22329258	glycosyl hydrolase family 17 protein; gamma-glutamyltransferase	61059/10.04
	41	AT1G65590	1 h	2190547	glycosyl hydrolase family 20 protein	60014/8.67
	42	AT4G23820	C; 1 h; 1 d	15236625	glycosyl hydrolase family 28 protein	48636/8.66
	43	AT1G45130	C; 1 h; 1 d *	15231354	glycosyl hydrolase family 35 protein; beta-galactosidase 1	81444/8.37
	44	AT5G26000	C; 1 h; 1 d	24417252	unknown protein; similar to glycosyl hydrolase family 3 protein	61133/5.70
	45	AT1G11840	1 h	15221116	glyoxalase 1 homolog; lactoylglutathione lyase	31929/4.91
cell wall modification	46	AT1G69530	C; 1 d	1041702	ATEXPA1 expansin A1	29280/9.09
	47	AT1G20190	C; 1 h; 1 d	15223799	ATEXPA11 expansin A11	26761/8.72
	48	AT5G63180	C; 1 d *	10177299	pectate lyase	46119/6.13
	49	AT4G24600	1 d	4220512	pectate lyase, putative	45037/8.04
	50	AT5G53370	C; 1 h; 1 d	15231828	pectin methylesterase 3/2; ATPME3	64256/9.24
	51	AT5G62350	C; 1 d	15241799	pectin methylesterase inhibitor family protein; invertase	22450/9.21
	52	AT3G14310	C; 1 h; 1 d	1932927	pectin methylesterase, putative	10678/10.16
	53	AT1G53830	C; 1 h; 1 d	2895510	pectin methylesterase, putative	64256/9.22
	54	AT5G45280	C; 1 h; 1 d	18415308	pectinacetylesterase, putative	42126/9.43
	55	AT2G45220	C; 1 h; 1 d	18406733	pectinesterase family protein	55977/9.37

signalling	56	AT3G02260	1 d	30678519	calossin-like protein required for polar auxin transport	574541/5.65
	57	AT5G23400	1 h *	15237312	disease resistance family protein; LRR family protein	64019/10.21
	58	AT2G26380	1 d	21536600	disease resistance protein, putative	52430/10.02
	59	AT1G33600	C; 1 h; 1 d	15217593	leucine-rich repeat family protein	52472/9.27
	60	AT3G20820	C; 1 h; 1 d	15232373	leucine-rich repeat family protein; polygalacturonase inhibitor-like	39863/8.50
	61	AT1G33590	C; 1 h; 1 d	10998936	leucine-rich repeat protein related hypothetical protein	51742/9.23
	62	AT5G12940	C; 1 h; 1 d	14532722	unknown protein; leucine-rich repeat family protein	39953/9.90
other cellular metabolic processes	63	AT1G29670	C; 1 h; 1 d	15220514	GDSL-motif lipase/hydrolase family protein	39872/8.63
	64	AT4G21280	C; 1 d	4583542	16 kDa polypeptide of oxygen-evolving complex	22991/9.64
	65	AT1G22840	1 d	15219956	ATCYTC-A/CYTC-1 (cytochrome C-1); electron carrier	12500/9.34
	66	ATCG00480	C; 1 d	7525040	ATP synthase CF1 beta subunit	53957/5.38
	67	AT1G42970	C; 1 d *	336390	glyceraldehyde 3-phosphate dehydrogenase B subunit	43168/5.60
	68	AT2G39730	C; 1 d	15450379	rubisco activase	52405/5.69
	69	ATCG00490	C; 1 h; 1 d	1944432	ribulosebisphosphate carboxylase; RBCL	52956/6.24
	70	AT2G22230	1 h *	18399910	beta-hydroxyacyl-ACP dehydratase, putative	24242/8.75
	71	AT5G17920	C; 1 d	55670112	cobalamine independent methionine synthase chain A; MetE	84283/6.02
	72	AT4G29210	C; 1 d	3080433	gamma-glutamyltransferase; putative	61191/10.06
	73	AT2G38540	C	15224899	nonspecific lipid transfer protein 1	11755/9.09
	74	AT2G10940	1 d	18396941	lipid transfer protein/ protease inhibitor family protein	30370/9.52
	75	AT3G06860	C; 1 h; 1 d *	15235527	multifunctional protein; ATMFP2; AIM protein	77859/9.89
	76	AT5G62330	1 d	8809646	similar to invertase/pectin methylesterase inhibitor family protein	22883/7.55
	77	AT4G28090	1 d	15234381	SKS10 (SKU Similar 10); copper ion binding oxidoreductase	62326/9.65
	78	AT1G76160	1 d	15222981	SKS5 (SKU Similar 5); copper ion binding oxidoreductase	60174/8.49
	79	AT1G41830	C; 1 h; 1 d	15234688	SKS6 (SKU Similar 6); copper ion binding oxidoreductase	60456/10.25
	80	AT4G34138	1 d	18418382	UDP-glucosyl transferase 73B1; abscisic acid glucosyltransferase	55257/5.51

unknown	81	AT1G46840	C; 1 d	15220125	F-box family protein	55412/9.39
	82	ATCG01000	1 d	7525093	hypothetical protein Arthcp087	214675/9.75
	83	AT1G28290	C; 1 h; 1 d	15217879	arabinogalactan protein 31; pollen allergen and extensin family protein	38489/10.84
	84	AT1G78820	C; 1 d	15219197	curculin-like lectin family protein; PAN domain-containing protein	51246/8.75
	85	AT3G15356	C; 1 h; 1 d	995619	lectin-like protein	29750/9.38
	86	AT5G03350	1 d	15242724	legume lectin family protein	30200/9.39
	87	AT3G28220	C; 1 h; 1 d	15232931	meprin and TRAF homology domain-containing protein	42887/8.80
	88	AT1G48090	1 d	30694236	protein kinase C2 domain containing protein	385059/5.76
	89	AT1G03230	C; 1 h; 1 d	18379072	extracellular dermal glycoprotein, putative	46148/9.46
	90	AT4G32460	C; 1 d	21537379	unknown protein	40215/6.92
	91	AT1G78830	C; 1 d	17644159	unknown protein	51007/8.74
	92	AT5G11420	C; 1 h; 1 d	15239049	unknown protein	39641/7.84
	93	AT5G25460	C; 1 h; 1 d	15239438	unknown protein	39978/7.44
	94	AT5G16780	1 d	13605837	unknown protein, F5E19_120	75072/5.31
	95	AT1G76780	C	15223730	unknown protein, heat shock protein-related	216902/4.51
	96	AT3G28830	C	11994791	unnamed protein product; similar to ATP binding	55501/10.42

Spot # represents arbitrary number given to each identified protein. Fraction found refers to the identifcation of the given protein in the corresponding sample type: C= control tissue; 1 h = 1 hour treatment at 4°C; 1 d = 1 day treatment at 4°C. Gene index number refers to GenBank GenInfo Identifier of identified protein sequence.

* Tubulin binding proteins identified by Chuong et al. 2004.

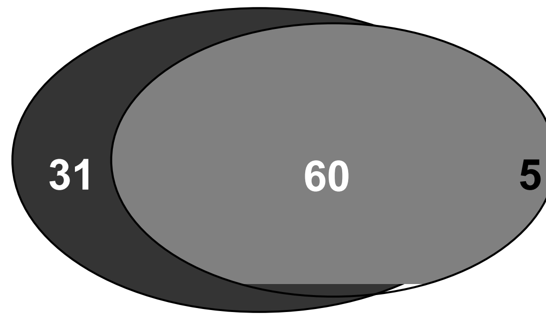

Figure 3.2. Venn diagram analysis of the tubulin binding proteins that differentially bind tubulin during 4°C temperature exposure. The majority of proteins were present in both sample types, while 5 where unique to control plants and 31 unique to low temperature treated plants.

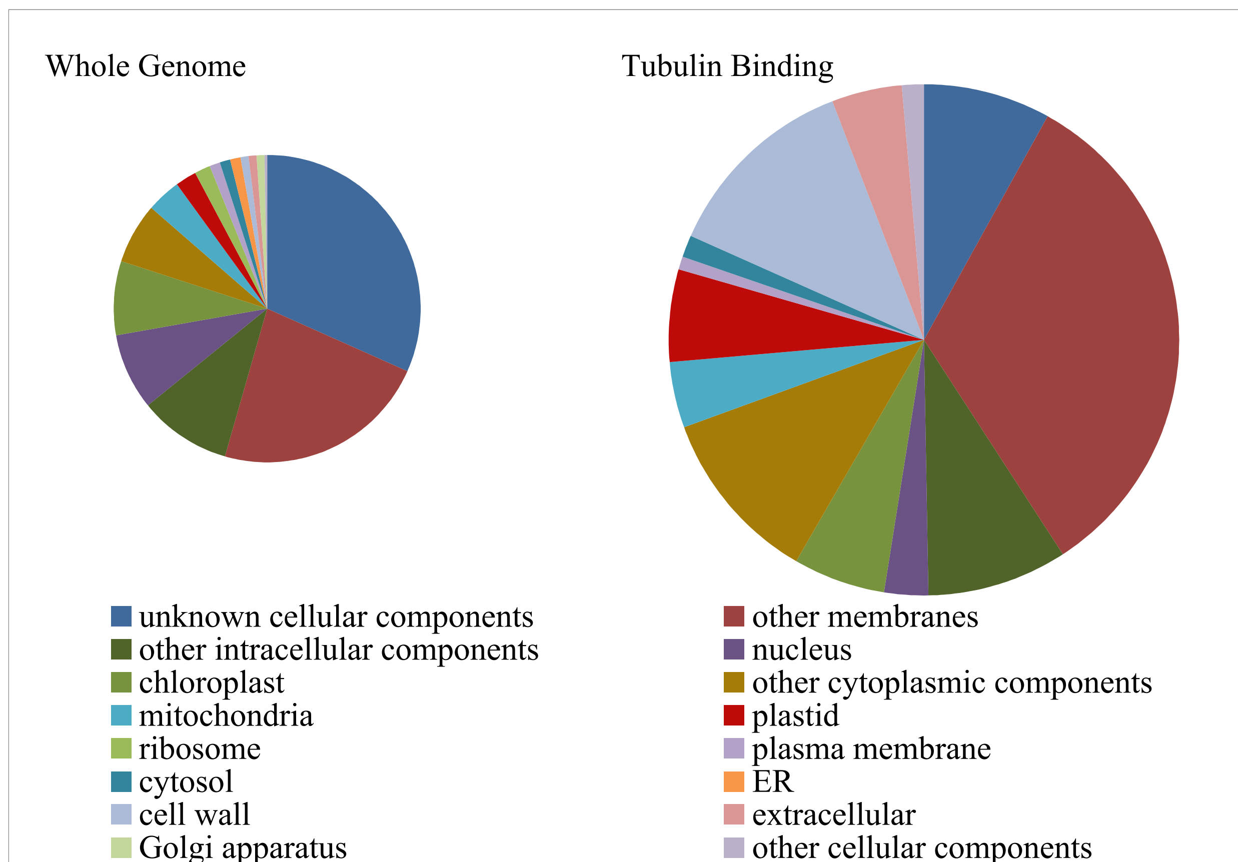

Figure 3.3. Classification of cellular component localization of identified tubulin binding proteins according to the gene ontology database. Classification of cellular component localization of proteins from the whole Arabidopsis genome is shown for comparison purposes (www.arabidopsis.org/idex/jsp).

Connections between MTs and endomembranes in plants have been demonstrated by organelle co-localization, and many MAPs were previously shown to have both endomembrane and cytoskeleton localization (Mathur et al. 2003; Cai et al. 2005). When compared with the annotation for the entire Arabidopsis genome, the tubulin binding protein fraction was enriched for cell wall proteins, which contributed to 12% of the tubulin binding protein fraction but less than 1% of the entire genome complement (Figure 3.3). The interaction between MTs and some of these cell wall proteins, including xylosidase, beta galactosidase, pectate lyase, and endoxylogucan transferase, has been described previously (Chuong et al. 2004).

3.3.2.2 Classification of tubulin binding proteins by putative function

Identified tubulin binding proteins were categorized by their known and predicted function (Figure 3.4). Proteins were classified into functional classes related to cell wall modification, carbohydrate metabolism, other cellular metabolic processes, RNA binding, protein metabolism, signalling, cell responses, and unknown function.

3.3.2.2.1 Proteins that modify the cell wall or carbohydrates. Proteins predicted to function in carbohydrate metabolism and cell wall modification comprised 23% of the identified tubulin binding proteins. Some of the proteins believed to modify cell walls include pectin modifiers, expansins, and proteins representing six families of glycosyl hydrolases (Table 3.1).

The majority of the hydrolases were identified in each temperature treatment type. However, a beta-xylosidase (spot 34) and beta-glucosidase (spot 30) (both family 3 glycosyl hydrolases) were found only in 1 d LT while a glycosyl hydrolase family 20 protein (spot 41) and glyoxalase 1 homologue (lactoylglutathione lyase; spot 45) were found only in the 1 h LT. Both expansin A1 (spot 46) and a pectin methylesterase inhibitor (spot 51) were found in control and 1 d LT tissue, but not in 1 h LT.

3.3.2.2.2 Proteins with other cellular and metabolic functions. A number of isolated tubulin binding proteins function in general metabolic and cellular processes. Some of these proteins act in photosynthesis and energy releasing pathways, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH; spot 67), a 16 kDa polypeptide of the oxygen-evolving complex (spot 64), rubisco activase (spot 68), ribulosebisphosphate carboxylase (spot 69), ATP synthase CF1 beta subunit (spot 66) and cytochrome C-1 (spot 65).

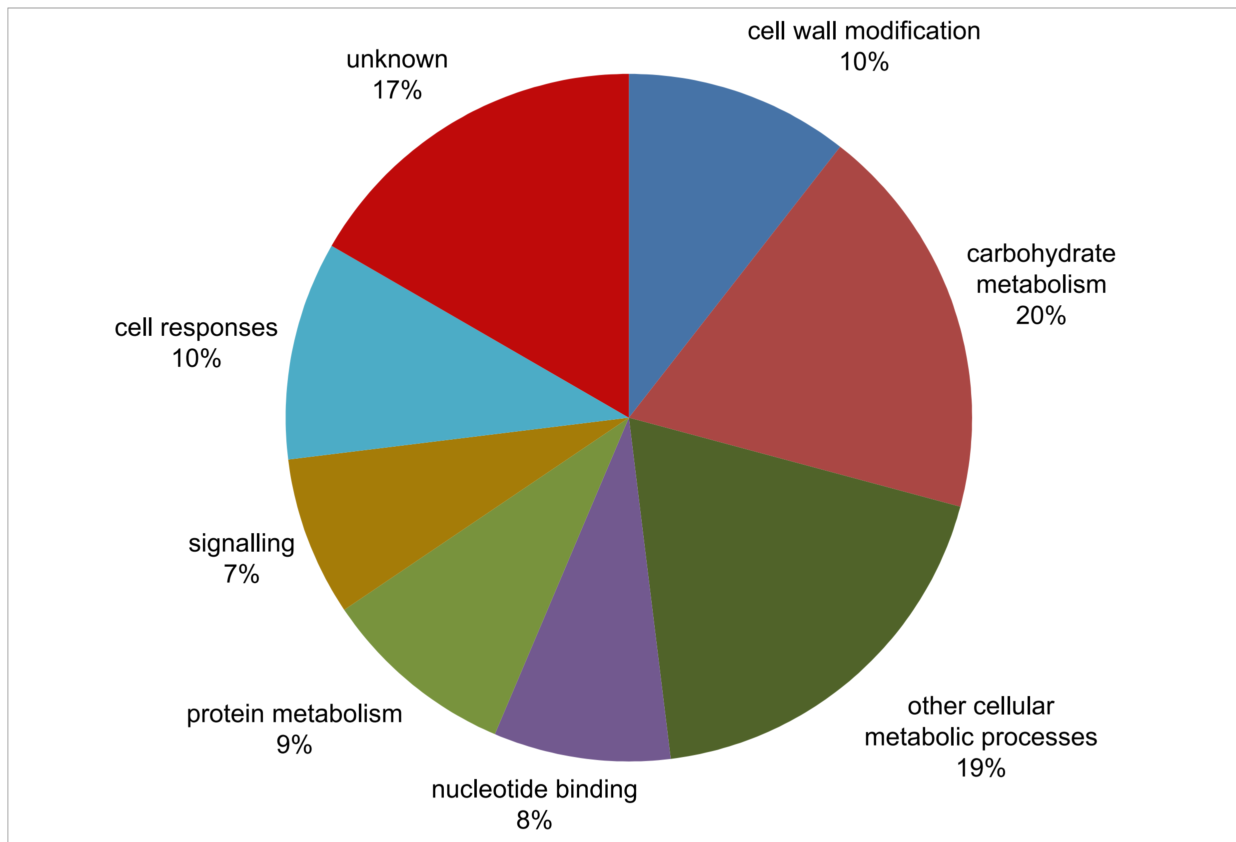

Figure 3.4. Functional classification of tubulin binding proteins identified by LC-MS/MS based n known (and putative) protein functions. Percentage values indicate the proportion of tubulin binding proteins present in the group.

Proteins active in amino acid biosynthesis and recycling (e.g. methionine synthesis enzyme; spot 71 and glutathione breakdown and amino acid recycling; spot 72), lipid metabolism (spots 63, 73, 74), and fatty acid metabolism (spot 70) were also identified. The identified peroxisomal multifunctional protein (MFP; spot 75) and one of the three copper ion binding oxidoreductases SKS proteins (spots 77-79) are well studied MT-binding proteins (Chuong et al. 2002; Sedbrook et al. 2004).

There was significant variability in binding of these proteins under the different temperature treatments. Four of the proteins were present in all three treatments and six were present in control and 1 d LT. There were also six proteins identified only in 1 d LT, and one protein unique to 1 h LT and control tissue.

3.3.2.2.3 RNA-binding proteins. Eight percent of the identified MAPs have nucleotide (RNA and/or DNA) binding capabilities. These include proteins that bind/modify tRNA (spots 7 and 8), a nucleoid DNA binding protein (spot 5), nuclear RNA-binding proteins (spot 3 and 4), a small nuclear ribonucleoprotein (spot 6), a glycine-rich RNA binding protein (spot 2), and a DEAD/DEAH-box RNA/DNA helicase (spot 1) (Table 3.1). The two tRNA associated/binding proteins and the glycine-rich RNA binding protein were only found in LT plants, while the helicase and one of the nuclear RNA binding proteins were only found in control plants.

3.3.2.2.4 Protein metabolising proteins. Proteins involved in protein metabolism and translation made up 9% of the isolated tubulin binding proteins. The RNA modifying pseudouridylate synthase protein (spot 17), involved in translation, was found in control and 1 d treatment while the well known tubulin binding protein, eEF1α (spot 10), was found in all treatment types. The remaining six protein metabolising enzymes were subtilase family proteins and aspartyl and serine proteases (spots 11-16).

3.3.2.2.5 Signalling proteins. Seven percent of proteins identified in the tubulin binding protein fraction were classified as signalling proteins. These include six disease resistance and leucine-rich repeat (LRR) family proteins (spots 57-62) and a calossin-like protein required for polar auxin transport (spot 56). The calossin-like protein and two disease resistance proteins were present only in LT plants.

3.3.2.2.6 Proteins responsive to stress and signals. Proteins annotated as responsive to hormones and other signals were identified in the tubulin binding protein fraction. Seventy percent of these proteins were found only in the low temperature treated samples. A thaumatin-

like protein (spot 27), similar to pathogenesis-responsive thaumatin proteins, was found only in the 1 d LT. Two proteins involved in hormone responses, a GA-responsive GAST1 protein homolog (spot 21) and a FAD-binding domain-containing protein (spot 20) were found in 1 d and 1 h LT tissue, respectively. Four proteins involved in protecting the cell against reactive oxygen species (ROS), a putative peroxidise (spot 22), two catalases (spots 18 and 19), and a serine hydroxymethyltransferase (spot 23), were identified in 1 d LT only. Three proteins classified as phosphate-responsive were identified in all samples. These include a spore coat protein-like protein with multi-copper oxidase (MCO) activity (spot 26) and two low phosphate responsive MCOs similar to the tobacco phosphate-induced (Phi)-1 and -2 proteins (spots 24 and 25).

3.3.2.2.7 Proteins with unknown function. No known biological function was ascribed to 16% of the proteins identified as tubulin binding proteins. Four of these proteins are lectins or lectin-like proteins (spots 83-86). An F-box family protein with possible involvement in mediating protein-protein interactions (spot 81) and a hypothetical protein (spot 82) are some of the remaining unknown proteins of this category.

3.4 Discussion

In this study, 96 proteins were identified as tubulin binding proteins using tubulin affinity chromatography and LC-MS/MS. Tubulin affinity chromatography was previously demonstrated to be a reliable method for isolating MT and tubulin-binding proteins (Durso and Cyr 1994; Chuong et al. 2002). The use of control columns, the consistent patterns of protein banding observed in replicates, and the identification of previously described tubulin and MT-binding proteins (e.g. ATMFP, eEF1α, SPR1) indicated that the tubulin-binding protein isolation was effective and reproducible.

3.4.1 Comparison to Previously Isolated Tubulin Binding Proteins

Although the total number of proteins identified in this study was similar to the number of tubulin binding proteins identified by Chuong et al. (2002), only 21% of these proteins were also present in the previous analysis. Differences of a similar magnitude have been described for other proteomics studies. For example, in five recent studies of the Arabidopsis cell wall proteome, only 30% of 268 cell wall identified proteins were present in at least two of the five studies (Boudart 2005; Bayer et al. 2006; Jamet et al. 2006). Further, when proteins identified by

LC-MS/MS were compared to proteins identified from the same samples run on 2D gels, groups of proteins were identified by only one technique or the other (Koller et al. 2002). Although LC-MS/MS is a much more sensitive technique, it appears unable to identify the full set of proteins identified by gel based techniques and therefore, many researchers consider these two methods complementary to each other (Koller et al. 2002). The relative little overlap in proteins identified in similar studies show that experimental design has significant impact on protein identification and proteome level biological responses. The tubulin binding proteome is likely larger and more complex than studies have currently revealed and further research should uncover yet additional proteins.

A major difference between the results of this study and those of Chuong et al. (2004) is the large number of cell wall modifying proteins found here, probably due to the different tissues used for protein extraction. A previous analysis by Jamet et al. (2006) found that significantly higher numbers of well known cell wall proteins (e.g. glycosyl hydrolases) were present in rosette leaves and etiolated hypocotyls compared to cell cultures. This difference in number is probably a related to the reduced wall differentiation in cell cultures compared to full plants (Jamet et al. 2006). The presence of fewer cell wall proteins within cell suspensions could also be a result of a loss of some extracellular proteins and other loosely bound cell wall proteins to the cell culture medium rather than being retained in the cell wall (Feiz et al. 2006).

3.4.2 Protein Localization

When examining the predicted cellular localization of identified tubulin binding proteins, the protein distribution largely corresponded to that of functional MTs throughout the cell. Tubulin binding by proteins that localize within various membrane-bound organelles such as mitochondria and plastids may at first seem surprising considering the cytoplasmic localization of MTs. However, proteins can have multiple functions corresponding to multiple sites of localization throughout the cell. Also, binding of these ‘organelle-localized’ proteins to tubulin may reflect the ability of MTs to distribute organelles and proteins throughout the cell (Romagnoli et al. 2003). Although actin filaments (AFs) are generally considered to be responsible for the transportation and localization of secretory vesicles and organelles throughout the plant cell, there is accumulating evidence indicating that some organelles can be transported via MTs, and that MTs cooperate with AFs to control secretory vesicle transportation and localization (Romagnoli et al. 2003; Wasteneys and Galway 2003; Romagnoli et al. 2007). The

molecular characteristics of the relationship between these two systems are not well understood; it is possible that features of this relationship would be revealed by examining endomembrane localized tubulin binding proteins identified in this study (e.g. some expansins and FAD-binding domain-containing protein).

Because cortical MTs underlie and communicate with the plasma membrane and the cell wall, proteins associated with both of these structures were expected in the tubulin binding fraction (Baluska et al. 2003). Many wall proteins were identified, but relatively few proteins known to localize within the plasma membrane were found (Figure 3.3). The identification of some of these proteins may have been compromised by the protein extraction techniques used, and using techniques that specifically enhance plasma membrane disruption may enable identification of more PM - MAPs (Santoni 2006).

3.4.3 Protein Functions

Proteins with a range of predicted cellular functions were identified as tubulin binding proteins in this study (Figure 3.4). The functions of some of the proteins are closely related to MT functions, including proteins involved in cell elongation. However, the majority of the identified proteins are involved in functions unrelated to MT and tubulin activity, and the purpose of many of these associations is as yet unknown. There is evidence supporting the involvement of some of the identified MAPs in the plant cold stress response. Proteins classified into functions ranging from stress responsive and signalling, to protein metabolism and other metabolic and cellular functions could play critical roles in stress sensing and signalling and increasing plant cold hardiness.

3.4.3.1 Microtubule-related functions

As previously mentioned, many important interactions occur between the cell wall and MTs. Cell elongation is one cell function that requires such interactions. MTs are involved in controlling cellulose microfibril orientation and seem to cooperate with a variety of cell wall proteins/enzymes to carry out their functions (Wasteneys and Galway 2003; Yoneda et al. 2007). Mutant plants with impaired MT organization or altered sensitivity to MT-specific drugs often display isotropic cell expansion and lose control of growth polarization (Burk et al. 2006; Furutani et al. 2000; Wasteneys and Galway 2003; Whittington et al. 2001). Pectin modification, e.g. via methylesterases, are important for plant development and cell growth, and tubulin binding glycosyl hydrolases help to control plant cell elongation and affect MT orientation

(Akashi and Shibaoka 1991; Takeda et al. 2002). Enzymes and cell wall components are continually being transported across the plasma membrane to permit growth, loosening, and elongation of the cell wall, and MTs may assist with these processes.

The close physical association between the cell wall, plasma membrane and MTs suggests that, at a minimum, changes to the cell wall will be transmitted to underlying MTs (Cyr 1994). When the cell wall arrangement is altered and turgor pressure causes the cellulose MFs to separate, the underlying MTs that are not immediately in line with the cellulose MFs must disassemble or break. The most sensitive component of the growing cell to strain or stress is believed to be the xyloglucans that connect the cellulose MFs to one another (Carpita and Gibeaut 1993; Cyr 1994). When put under strain, such as that occuring during cell expansion, the xyloglucan chains and MTs must yield, by breaking or disassembling, in order to allow relaxation of cell walls and subsequent cell growth. Many of the identified tubulin binding carbohydrate metabolising and cell wall modifying proteins, including the expansins and many of the glycosyl hydrolases, are believed to participate in cell elongation by loosening the cell wall and disrupting the xyloglucan bonds holding cellulose fibrils together (Van Sandt et al. 2007).

Altered activity of cell wall/carbohydrate modifying proteins will change the state of the cell wall to either promote or suppress cell elongation. Many of the wall/carbohydrate modifying proteins had altered MT-binding at the different temperature treatment points (Table 3.1). Because plants have reduced growth and undergo cell wall modifications during cold acclimation, changes in cell wall modifying proteins should reflect changes in wall metabolism occurring at these time points. Many of these cell wall modifying enzymes respond to hormones, light, cold and other external stimuli, which all affect MT orientation, cell growth and elongation (Shibaoka 1994; Xu et al. 1996; Khokhlova et al. 2003; Le et al. 2005). Possibly, cell wall/ carbohydrate modifying proteins act in conjunction with MTs as part of a complex system involved in responding to elongation signals, controlling cell wall loosening, orienting the cellulose synthase complex and determining the general state of cell expansion. Pectin modifications (e.g. deesterification by pectin methylesterase) control the effectiveness of other cell wall enzymes, are important in the plant defence response, and could directly affect plant cold tolerance (Lionetti et al. 2007).

Three SKS proteins were identified as tubulin binding proteins in this study. Despite being annotated as having copper ion binding and oxidoreductase activity, these SKS proteins have been shown to lack characteristic copper binding motifs and have no clear enzymatic function (Sedbrook el al. 2002). One of these proteins, SKS6, was identified in each of the temperature treatments. SKS6 (a.k.a. SPR1) is a well-known plant MAP important for cell expansion and directional cell growth (Furutani et al. 2000; Sedbrook et al. 2004). SPR1 is believed to act as an intermolecular linker that controls axial twisting and touch-induced directional cell expansion (Sedbrook et al. 2004). The other two SKS proteins were identified only in the 1 d LT. Although not previously described as MAPs, these proteins may have similar or related functions.

3.4.3.2 Non-microtubule functions: possible microtubule involvement?

Proteins involved in processes apparently unrelated to MT function made up the largest proportion of tubulin binding proteins. The importance of the protein-MT association of each one of these proteins is not known, but there is evidence indicating that some of these associations are important for normal, efficient cell metabolism and function. While evidence supporting these interactions in animal systems is abundant, only one example of such associations has been clearly demonstrated in plants. RNA metabolism and transportation involves the association of RNA with the cytoskeleton (Jansen 1999; Chuong et al. 2002; von Groll et al. 2002). Evidence dating from the early 1990’s show that polysomes associate with the plant cytoskeleton via interactions between MAPs and mRNA (Davies et al. 1991). RNA binding proteins, including small nuclear ribonucleoproteins, helicases and other RNA-binding proteins, were identified in the tubulin-binding protein fraction of this study as well as that of Chuong et al. (2004). RNA binding proteins and other proteins involved in translation could assist with both of these functions, ensuring efficient translation and transportation of mRNA throughout the cell.

In animals, many tubulin folding cofactors and MAPs (e.g. CLIP-170s) are glycine-rich proteins (GRPs). Glycine-rich cytoskeleton-associated protein domains are responsible for mediating the association of these proteins with MTs (Mishima et al. 2007). Direct associations between GRPs and MTs in plants have yet to be defined, but many GRPs have functions such as controlling cell wall constituents and other MT related functions (Mousavi and Hotta 2005). DNA and RNA helicases are involved in many cellular functions, including DNA and RNA repair and stability, the regulation of mitosis, and posttranscriptional gene silencing (Jacobsen et

al. 1999). It is thought that helicases can mediate some interactions between the cytoskeleton and RNA processing and transport (Zhang et al. 2002).

Proteins with functions ranging from energy releasing pathways and photosynthesis to lipid metabolism and amino acid and fatty acid biosynthesis were identified in this study as MAPs. Although the purpose of MT binding has not been demonstrated, the isolation of some of these proteins supports previous evidence of MT binding. The Calvin cycle enzyme GAPDH (spot 67), is known to bind to MTs and affect MT bundling in both animals and plants (Somers et al. 1990; Chuong et al. 2004). Enzymes involved in catalyzing methionine synthesis, such as spots 71 and 72, were previously shown to associate with MTs and secretory vesicles in pollen tubes (Moscatelli et al. 2005). The association of multifunctional protein ATMFP2 (spot 75) with MTs has been well-studied in plants. Similar to the MT binding of RNA binding proteins, ATMFP binds to MTs to facilitate its functioning in the transport of peroxisomes, mRNA transport and translation (Chuong et al. 2002). The reason for MT binding has been examined in very few proteins, but where studied, MT binding was found to play an important role in protein function and transport (Jansen 1999; Chuong et al. 2002; Lopez-Valenzuela et al. 2003). One can therefore expect that the association of some proteins with MTs is a requirement for their function, even if at this moment in time that function is unknown.

Some RNA associated proteins and proteins involved in various cellular and metabolic processes appear to be stress responsive. Differences in MT binding of some proteins in response to low temperature treatments was observed. Rubisco activase, cytochrome C-1, glyceraldehyde 3-phosphate dehydrogenase and the ATP synthase CF1-beta subunit have all been previously identified as cold responsive, and RNA associated proteins seem to be important to the cold response (Chuong et al. 2002; Gong et al. 2002; Hannah et al. 2005; Dong et al. 2006; Yan et al. 2006). RNA helicases act as abiotic stress response repressors and positive regulators of *CBF* gene expression, while nucleotide binding proteins are important in stress responses (Gong et al. 2002; Kant et al. 2007). Glycine-rich RNA binding proteins have been implicated in several physiological processes (Mousavi and Hotta 2005) and the glycine-rich RNA-binding protein 7 in Arabidopsis (ATGRP7) was identified in 1 d LT plants. ATGRP7 is a circadian clock regulated protein and *atgrp7* mutants are hypersensitive to ABA and osmotic stress conditions (Cao et al. 2006). It is unclear what generates the stress sensitivity in *atgrp7* mutants, or if the

process is ABA-dependent or -independent (Cao et al. 2006), but in either case MT binding may have a role in facilitating the stress response.

Four of the identified tubulin binding proteins where annotated as multi-copper oxidases (MCOs). The calossin-like protein (spot 56) identified in 1 d LT plants is a MCO with ubiquitin-protein ligase activity. Plants encoding mutant forms of this protein display altered photomorphogenesis and defects in auxin responses and auxin transporter localization (Gil et al. 2001). The remaining three proteins with MCO activity respond to low phosphate signals. The spore coat protein-like protein (spot 26) MCO appears to be involved in reprogramming cell growth in roots following contact with low phosphate media, by sensing the stress and initiating signals to stunt primary root growth (Svistoonoff et al. 2007). Two phosphate responsive MCOs are closely related to the phosphate-induced (Phi)-1 and -2 bZIP-type transcription factors originally identified in tobacco (Sano et al. 1999; Sano and Nagata 2002). *Phi-1* and *Phi-2* transcription is rapidly induced following an application of external phosphate or ABA, or changes in cytoplasmic pH, with maximum transcript levels obtained after approximately 1 h (Sano et al. 1999; Sano and Nagata 2002), and the potato *Phi-1* orthologue was identified in the potato abiotic stress transcriptome (Rensink et al. 2005), suggesting a role for this protein in multiple abiotic stress responses. The electronic Fluorescent Pictograph (eFP) Browser (www.bar.utoronto.ca/efp), which visually displays microarray data of approximately 22,000 genes from Arabidopsis (Winter et al. 2007), as well as EST expression data, have both identified the gene encoding Arabidopsis Phi-1-like protein (At5g64260) as highly responsive to low temperatures (Kilian et al. 2007; Robinson and Parkin 2008). The cold induced expression and their similarity to transcription factors suggest that these proteins may act as stress induced transcription factors that bind to the MT cytoskeleton and support protein translocation to the nucleus where it encourages low temperature induced gene expression. Arabidopsis Phi-1-like protein was previously identified as a tubulin binding protein (Chuong et al. 2004). The SKS proteins, including the MAP SPR1, are structurally similar to these multiple-copper oxidases, and including the SKS proteins, seven copper-oxidases were identified as MAPs in this study. It would be interesting to identify conserved features of these proteins responsible for MT binding activity.

A non-annotated F-box family protein (spot 82) bound to tubulin in 1 d LT and control treatment. F-box domains generally function to mediate protein-protein interactions including

polyubiquitination, centromere binding, translational repression and transcription elongation. In plants, many F-box proteins are transcription factors controlling various stress responses. A group of plant F-box proteins (with unknown functions), up-regulated by cold, have orthologues in animals. This group of animal proteins are transcription factors that shuttle between the plasma membrane and the nucleus in response to environmental cues (Lai et al. 2004). The Arabidopsis eFP database indicates that gene expression of the F-box protein is nearly doubled after 1 h at LT before returning to base levels. Possibly after exposure to LT, the identified F-box family protein follows a similar pathway as described for animal F-box proteins, where it is transmitted along MTs from a site of stress sensing to the nucleus and upon release from the MTs, it functions to activate stress-responsive genes.

During exposure to low temperatures there are many changes in gene expression with new groups of cold responsive proteins being synthesized. Tubulin has been shown to cross the nuclear membrane following low temperature exposure (Chapter 3; Schwarzerová et al. 2006), possibly as a result of interactions between tubulin binding proteins and proteins of the NPC that are important in stress responses (Xu and Meier 2008). The localization of tubulin and tubulin binding proteins (e.g. F-box family protein and Phi-1-like) to the nucleus could possibly affect gene expression and the subsequent transport of mRNA out of the nucleus.

3.4.3.3 Proteins involved in stress sensing, signalling, and cold tolerance

Proteins annotated as responsive to various signals and hormones comprised 10% of the identified tubulin binding proteins. Four of these proteins (spots 18, 19, 22 and 23) were identified only in low temperature treated samples and function to protect the cell against ROS. ROS are produced under various stress conditions and can have two different outcomes: they can function as secondary messengers that affect the activity of various proteins leading to a variety of cellular responses, while excess ROS production can cause oxidative damage to cellular compartments. The presence of antioxidant proteins only in 1 d LT may reflect a need for the activity of these proteins to remove excess ROS and avoid damage caused by low temperature-induced, lowered photosystem reaction rates.

Two hormone responsive proteins (spots 20 and 21) were found in 1 d and 1 h LT tissues. Plant hormones are known to elicit changes in MT structure and dynamics, and as such, are important in stress responses and low temperature tolerance (Davies et al. 1991; Chuong et al. 2002; von Groll et al. 2002). There are many ways that hormones may require or make use of

MTs to facilitate plant stress responses. Hormones, such as auxin and GA, affect plant growth by controlling cell elongation, such that the presence of hormone responsive proteins on MTs in cold-treated plants may reflect a conserved function related to reducing cell growth/elongation following exposure to different stimuli. Thaumatin proteins are involved in pathogenesis-related responses, and the presence of the thaumatin-like protein (spot 27) in 1 d LT plants suggesting a possible general stress response protein function.

Many MAPs not annotated as stress/signal responsive are capable of responding to various stimuli. For example, the protein kinase C2 domain containing protein (spot 88) has an unknown biological function but has characteristics that link it to stress responses and MTs. These include a Ca^{2+}-binding motif found in protein kinase C and identity with proteins containing a pleckstrin homology (PH) domain, common in MAPs and proteins involved in intracellular signalling (Lemmon and Ferguson 2000). The kinase C2 domain protein was only identified in 1 d LT tissue, indicating a possible increased MT-dependent activity during prolonged cold exposure. The meprin and TRAF homology domain-containing protein (spot 87) also has no annotated function or cellular localization, but it was identified as being highly upregulated in response to salt stress, suggesting another link between tubulin binding proteins and common stress responses (Gong et al. 2001).

Six disease resistance and leucine-rich repeat (LRR) family proteins (spots 57-62), described as signalling proteins, were found to interact with tubulin in this study. Plant proteins containing LRR domains are important to various plant-environmental and developmental signal transduction pathways (Osakabe et al. 2005). The LRR domain functions as a hormone receptor capable of linking biotic, abiotic, and ABA signalling pathways (Osakabe et al. 2005). LRRs are the primary domain responsible for recognizing pathogens and controlling the specificity of plant defence mechanisms (Banerjee et al. 2001). LRRs perceive signals and mediate intracellular interactions among proteins, and the binding to MTs may help facilitate this signalling process. Beyond stress sensing and signalling function, many LRR proteins exhibit high sequence similarity to antifreeze proteins that act to modify ice crystal formation (Meyer et al. 1999). Two of the disease-resistance LRR proteins were found only in cold treated plants, indicating a potential role in the cold response.

Two lipid transfer proteins (LTPs) potentially involved in cold acclimation and increasing cold hardiness were found in this study. LTPs are usually small, extracellularly

located proteins with a range of proposed biological functions that can act to bind acyl chains and enhance the transfer of phospholipids between membranes. Some LTPs function as cryoprotective proteins and LTP overexpression can increase plant cold tolerance; seven genes encoding LTPs are upregulated in the cold tolerant Arabidopsis *esk1* mutants (Bubier and Schlappi 2004; Xin et al. 2007). A LTP family protein/protease inhibitor protein (spot 74), found only in 1 d LT tissue, contains a protease inhibitor domain and lipid transfer domain capable of binding to and transporting membrane lipids. It is proline-rich and shares significant sequence similarity with cell wall-plasma membrane linker proteins, extensin-like proteins, and other LTPs previously shown to increase Arabidopsis freezing tolerance (Bubier and Schlappi 2004). Its presence only in 1 d LT tissue supports a possible role for this LTP in freezing tolerance.

The nonspecific LTP1 protein (spot 73), found only in untreated Arabidopsis samples, is a calmodulin binding protein involved in the calmodulin-signalling pathway (Wang et al. 2005). Two different classes of proteins that interact with tubulin and bind to calmodulin have been described in plants; those in which tubulin binding requires Ca^{2+}, and those that only bind tubulin when Ca^{2+} levels are low (Zielinski 1998). The latter group appear to act in conjunction with calmodulin to stabilize microtubules (Zielinski 1998). Low temperatures increase intracellular Ca^{2+} levels, which would cause proteins of the latter class to release from tubulin. Nonspecific LTP1 was not identified as a tubulin binding protein in low temperature exposed material, indicating it likely is a low Ca^{2+} specific binding LTP. Therefore, once released LTP1 would be unable to stabilize MTs, thereby encouraging low temperature-induced MT disassembly.

Four lectins/lectin-like proteins (spots 84-87) were identified in this study. While the role of lectins in normal plant functioning is relatively poorly understood, recent studies suggest a possible involvement in the plant cold stress response. Once thought to only act as storage proteins, changes to lectin protein content and activity in response to low temperatures and over the course of cold acclimation suggests that they are involved in plant stress responses (Sonobe et al. 2001; Peck 2005; Garaeva et al. 2006). Further, they have been shown to colocalize with the cytoskeleton and their behaviour is dependent on the integrity of the cytoskeleton (Wasteneys and Galway 2003; Garaeva et al. 2006; Nguema-Ona et al. 2007). The curculin-like lectin (spot 85) may act as a link between the cytoskeleton and vesicle membranes and to control plant growth in response to red light (www.ebi.ac.uk/interpro/databases.html). This lectin was only present in control and 1 d LT tissue, possibly reflecting a separation of membranes and

cytoskeleton during the early stages of cold acclimation. Unlike curculin-like, legume-like lectin (spot 87) was only identified in 1 d LT. This protein shares features with many predicted protein kinases suggesting a potential involvement in signalling. Arabinogalactan proteins (AGP) (spot 84) are also lectin proteins and are the best candidates, of the lectins, for linking MTs to the plasma membrane and MT activity to signalling sensing and responses (Peck 2005). Plants mutated in certain AGP proteins have altered stress sensitivity, thin cell walls and inhibited cell expansion, resembling various cytoskeleton mutants (Shi et al. 2003).

The stress sensing and signalling potential of AGPs and lectin-like proteins is partially attributed to the presence of glycosyl phosphatidylinositol (GPI) anchors on these proteins. In addition to lectins and AGPs, glycosyl hydrolase family 17 proteins, lipid transfer-like proteins, SKS proteins, and pectate lyase-like proteins, all identified as tubulin binding proteins, are thought to be GPI anchored proteins (Borner et al. 2003; Elortza et al. 2003). As mentioned in Chapter 1, GPIs are added posttranslationally to the C-terminal ends of certain proteins and act to anchor these proteins in the membrane in a manner different to that of transmembrane protein anchoring. While anchored in the plasma membrane, many GPI anchored proteins (GAPs) are actually cell wall localized proteins. However, these proteins can become solely cell wall localized if the GPI domain is cleaved off by the activity of PLD (Borner et al. 2002). Disruption of some GAPs has been shown to promote MT disassembly. Therefore, MT rearrangement, induced by PLD, may occur as a result of GPI anchor cleavage (Nguema-Ona et al. 2007). GPI anchors may function to maintain proper localization or function of the anchored protein, and cleavage of the GPI results in alterated protein function and/or interactions with other proteins (Borner et al. 2002). With their role in extracellular matrix remodelling and signalling, GAPs may act as important connectors between the extracellular matrix and the cytoskeleton (Baluska et al. 2003). Moreover, both MTs and GAPs localize to membrane microdomains believed to act as platforms for cellular trafficking, cell wall metabolism and signalling and stress responses (Borner et al. 2003). Proteomic studies of GPIs, GAPs and membrane microdomains in plants are in their infancy (Borner et al. 2003; Grennan 2007); however, it is highly likely that future research will reveal MT and MAP functions closely connected to these elements.

Subtilase family proteins and aspartyl and serine proteases (spots 11-16) are stress and pathogen responsive proteases believed to be involved in development, protein turnover, and signalling cascades (Rautengarten et al. 2005). At least one of the subtilase-like proteins is

believed to localize to both the apoplast and plasma membrane and to function in generating extracellular signals required for cell-to-cell communication (von Groll et al. 2002). Binding of six of these protease proteins to tubulin was affected differently by low temperature treatments (Table 3.1). Interestingly, subtilases are known to modify tubulin by cleaving (shortening) the β-tubulin C-terminal end (Bokros et al. 1996), which could have significant consequences on MT stability, as it is the C- terminal end of β- tubulin that is responsible for MT cold-sensitivity. Removal of this domain by subtilases increased the cold stability of MTs containing these modified tubulin proteins (Bokros et al. 1996), therefore, subtilases may act on tubulin during the cold acclimation process to increase MT cold stability.

The cytoskeleton is present throughout the cell, making up a large surface area that interconnects nearly all cell structures. This large and highly charged MT surface area provides a site of localization and stabilization for all kinds of cytoplasmic components. The identification of diverse types of tubulin binding proteins supports the idea that many proteins use the cytoskeleton as a scaffold to facilitate their transport and interaction with other cellular elements. Additional research is required to determine if the proteins identified in this study interact with MTs or associate with other MT-binding proteins in planta.

The predicted functions and expression patterns of tubulin binding proteins identified in control and short term cold acclimated Arabidopsis plants has revealed numerous and diverse ways that MTs may be involved in the Arabidopsis low temperature response. Further investigation (Chapter 4) will provide more detailed insight into the role of these MAPs in the low temperature response in Arabidopsis, and how these proteins are affected by low temperature.

CHAPTER 4
GENERAL DISCUSSION AND FURTHER RESEARCH

4.1 General Discussion and Conclusions

In this work several aspects of the relationship between low temperature stress and plant MTs in Arabidopsis were investigated. Despite abundant evidence in the literature suggesting that the cytoskeleton may be involved in the plant response to low temperature stress only a few studies have attempted to directly examine this link. One highly cited publication indicated that MT reorganization is able to initiate the cold acclimation process (Abdrakhamanova et al. 2003). However, there has been no follow up work since its publication nearly five years ago, leaving the mechanism by which MTs initiate cold signalling unknown and questioning if a similar process occurs in other species.

In the first part of this project the effect of low temperatures on MT organization in Arabidopsis were monitored and the potential consequences of these changes examined. In this study the changes to MT organization caused by low temperatures were visualized in a live plant system for the first time. MTs were observed undergoing transient partial disassembly when exposed to cold acclimation temperatures, similar to the rearrangements observed in immunolabelled root MTs in cold tolerant winter wheat cultivars exposed to cold acclimation treatment (Abdrakhamanova et al. 2003). However unlike previous results, disassembly and reassembly of MTs was found to be insufficient to increase cold hardiness to the full extent of cold acclimation in Arabidopsis (Chapter 2). The results of this study support the idea that changes to MT composition, not induced by MT disruption with chemicals, such as altered MAP binding, are necessary for complete cold hardiness in plants.

The second part of this project was a study of tubulin binding proteins present in untreated and cold treated Arabidopsis tissue. The MT cytoskeleton is an important site for the binding of many proteins, but likely only a portion of proteins that make up the plant MT binding proteome have been identified to date (Chuong et al. 2004; Forgacs et al. 2004). Some of these proteins are critical to controlling MT activity and organization while the binding of others do

not appear to affect MTs. The large-scale analysis of tubulin binding proteins performed in this study has appreciably expanded the identification of tubulin binding proteins.

This was also the first analysis of the impact of exposure to stress on binding of proteins to MTs. Comparing the MT binding proteins found in 1 h and 1 d cold-treated plants vs. control plants provided information on how MTs may be involved in the plant response to low temperature stress. For example, signalling proteins that only bound to MTs in stressed plant tissue supports the concept of MTs serving as a track for signal transport. The cellular elements that are responsible for sensing low temperature signals and linking MTs and the cell wall are still unknown. The considerable presence of cell wall proteins in the tubulin binding proteome provides many candidate proteins that may link these elements and/or facilitate communication between extracellular matrix and MT cytoskeleton.

In conclusion, this project was successful in determining how MT arrays and MAPs respond to low temperatures. This work provides an extensive framework on which further analysis, to determine precisely how MTs act to increase plant cold hardiness, can be based.

4.2 Further Research

The general goals of this study were broad, and more complex than can be fully examined in the spectra of a Masters Thesis project. Cold acclimation and MAPs are both areas of research with lots of unknowns. There are many ways this study could be expanded to gain further insight into the relationship between MTs and the cold acclimation process in plants. Questions related to this topic, that arose during the course of this research and would benefit from further study, are discussed below.

4.2.1 Examination of Microtubule Disrupting Chemicals

In this study, pronamide and oryzalin treatments had very different effects on freezing tolerance, with the differences likely related to the different modes of action of the two chemicals. While there is some idea of how these chemicals cause MT disassembly, relatively little is known about what effect, if any, these chemicals have on the binding of MAPs and other cell constituents, or if these chemicals have preferential binding to different tubulin isoforms. Research should be carried out to answer these questions and examine the relationship between low temperature and chemically induced MT reorganization.

4.2.2 Examination of Tubulin Isoform Changes

Past literature indicates that there are tubulin isoforms with differing sensitivities to low temperatures. Combined with the observed gradual changes in MT arrangement and inconsistent effects of chemically induced MT disassembly on freezing tolerance, these results suggest that the shift from dynamic, cold-sensitive MTs to slower moving, cold-hardy MTs may be important to the acquisition of MT and plant freezing tolerance.

It has been reported that the C-terminal end of the tubulin protein is responsible for the sensitivity of MTs to cold induced disassembly. Post secondary modifications to tubulin, the high presence of acidic or basic amino acids, and binding affinity of MAPs to different protein motifs may alter MT dynamics or directly make MTs more sensitive to low temperature-induced depolymerization. Examining the features of the C-terminal domain that contribute to cold sensitivity would greatly enhance our understanding of the role of MTs in plant cold hardiness.

Future studies should be carried out to examine expression levels of tubulin genes and proteins during cold acclimation in Arabidopsis, to determine if MT disassembly during cold acclimation occurs in order to permit the formation of MTs with cold resistant tubulin isoforms. Creating and examining plant lines with altered expression levels of the cold sensitive and cold hardy tubulin isoforms, including over-expression and gene knockout (KO) lines, would provide considerable insight into the importance of MTs in cold hardiness.

4.2.3 Characterization of Tubulin Binding Proteins

In planning this study we predicted that many of the tubulin binding proteins involved in low temperature response would completely change their tubulin association following exposure to low temperatures. While some proteins with these characteristics were identified, most proteins were present in both control and low temperature-treated samples. Future studies to identify more quantitative changes in tubulin binding during low temperature exposure could use quantitative proteomics techniques such as differential in-gel electrophoresis (DIGE), isotope-coded affinity tagging (ICAT), and protein labelling using isobaric tags for relative and absolute quantification (iTRAQ).

A large subject of future research is a more in depth examination of the tubulin binding proteins identified in this study. The ability of these proteins to bind tubulin/MTs should be examined using techniques such as yeast two hybrid assays and fluorescent protein studies. Some of the identified proteins may interact with other MAPs and other proteins of interest, and the

full array of possible protein-protein interaction partners should be identified to establish mechanisms and outcomes of MT-interactions.

Other powerful tools for studying proteins at a functional level are mutant plant lines suppressing or over-expressing the proteins of interest. Over-expression and KO plant lines can be examined to determine the effect these proteins may have on MT activity and organization. The ability of these mutants to cold acclimate and perform low temperature signalling could also be assessed through means such as traditional LT_{50} determination.

CHAPTER 5
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APPENDIX
PRELIMINARY CHARACTERIZATION OF PUTATIVE MAPS

Preliminary characterization of three putative MAPs was undertaken to investigate their potential involvement in MT activity and low temperature responses. Two MAPs, a phosphate-responsive protein (phi-1-like, At5g64260; spot 25), and a glycosyl hydrolase family 3 protein (xylosidase, At5g49360; spot 35) were selected for analysis. A third protein (DRP2B, At1g59610) belonging to the dynamin-related family was also selected because it was identified among a group of cold-responsive nuclear proteins (Parkin, unpublished results).

GFP fusion proteins were created for each of the three target proteins and preliminary results reveal their potential localization patterns. Preliminary protein expression analysis of GFP:phi-1-like phosphate responsive protein-GFP fusion protein in tobacco leaf epidermal cells expression systems showed protein localization at the cell wall, plasma membrane, and cytoplasm (Figure A.1A and B). Fluorescence was limited to the cell periphery and what appeared to be the cell wall and plasma membrane in N-terminal GFP fusions (Figure A.1A), while C-terminal GFP fusion proteins were present throughout the cytoplasm (Figure A.1B) in punctate patterns streaming throughout the cytoplasm. The different localization patterns of the differently labelled proteins suggest that the presence of the GFP tag interferes with some part of the protein trafficking.

Fluorescence from GFP:DRP2B fusion proteins expressed in tobacco leaf epidermal cells was localized to the cell periphery and large vesicle-like structures at the cell periphery (Figure A.1C). The identity and significance of the fluorescent patches remains to be determined. Fluorescence from GFP:xylosidase expressed in tobacco leaf epidermal cells appeared to be concentrated in thickened areas of the cell wall, while Arabidopsis transformants showed fluorescence localized to the cell wall and globules within the vacuole of root epidermal cells (Figure A.1D). This observed vacuolar accumulation of GFP:xylosidase is unusual and of unknown significance.

More detailed/thorough analysis of protein expression is required to verify subcellular localization of these proteins. Analysis should include co-expression of the fusion proteins with

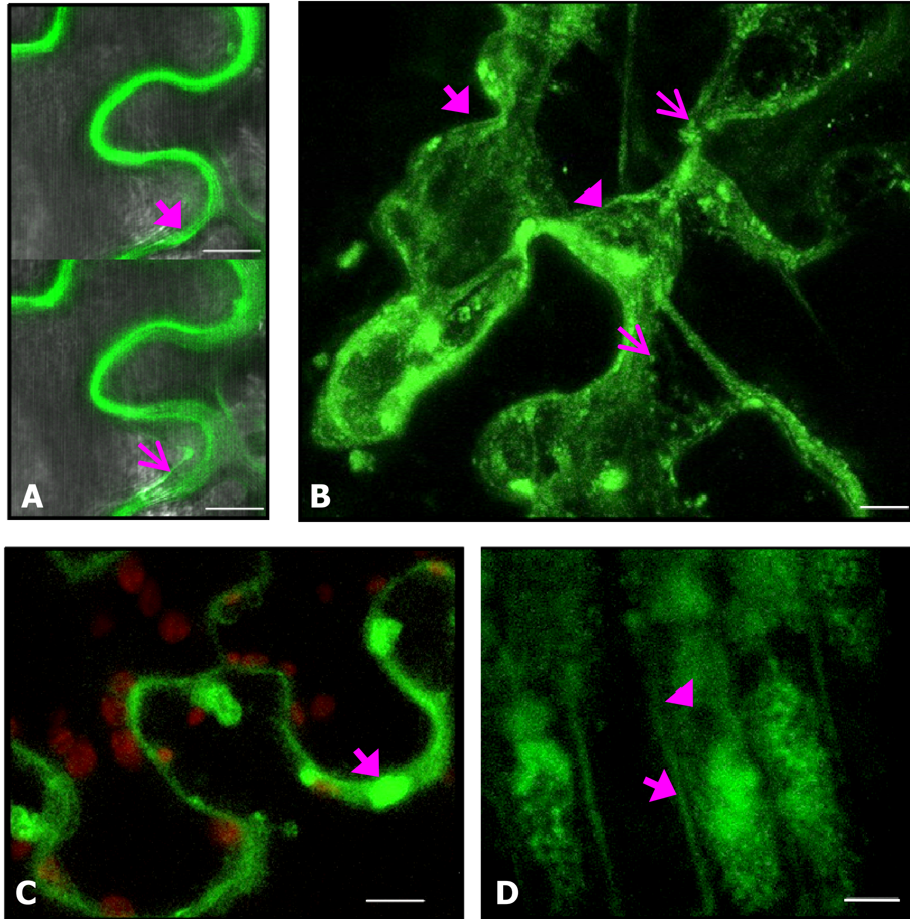

Figure A.1. Expression of GFP-target protein fusions revealing putative cellular localization. (A) Localization of phi-1:GFP and (B) GFP:phi-1 in tobacco leaf epidermal cells. (A) Tobacco cell at early stage of plasmolysis showing separation of the plasma membrane (open arrow) from the cell wall (closed arrow). (B) GFP:phi-1 fluorescence throughout the cytoplasm with some punctate distribution (open arrows). (C) Expression of GFP:DRP2B in tobacco epidermal cells with fluorescent accumulations at the cell periphery (closed arrow). Red indicates chloroplast autofluorescence. (D) Localization of GFP:xylosidase expressed in Arabidopsis root cells at the cell wall (closed arrow) and in bright accumulations within the vacuole. Arrowheads indicate location of nucleus. Scale bars = 10 µm.

fluorescent labelled organelle markers and cytoskeleton proteins to determine if the proteins co-localize with MTs or other organelles. Stable lines of Arabidopsis, expressing the MT markers together with the described fusion proteins, as well as lines expressing fluorescently labelled 5' regulatory regions of the protein's should be created to identify if the proteins co-localize with MTs and where and when the genes are expressed.

Preliminary examination of Arabidopsis SALK lines with gene KOs of xylosidase and the dynamin related protein was undertaken. Preliminary results suggest that control and homozygous SALK gene KO lines have no characteristic morphological differences, differential responses to ABA, pronamide or taxol, or differences in salt or freeze tolerance.

The presence of many duplicated and potentially functionally redundant genes in Arabidopsis can often limit the value of gene KO studies. A lack of detectable phenotype would result from a functional redundancy among members of the same gene family. The loss of gene expression in a KO line could be compensated for by increased expression of other members of the gene subfamily.

The xylosidase is a member of the glycosyl hydrolase family 3. There are 14 members of this family, and three Arabidopsis family members share >60% protein identity with xylosidase At5g49360. Creating a plant line with gene KOs of multiple members of this family may provide more insight into this protein function. One protein in Arabidopsis is similar to the Phi-1 phosphate induced protein, Phi-2. Although there are 16 dynamin-related proteins in Arabidopsis, only one protein in Arabidopsis, DRP2A, is closely related to DRP2B (Hong et al. 2003). The function of both DRP2B and Phi-1 would therefore be best examined in Arabidopsis lines containing double KOs of the respective closely related genes.