The Expanding Diversity of Plant U-box E3 Ubiquitin Ligases in *Arabidopsis*: Identifying AtPUB18 and AtPUB19 Function during Abiotic Stress Responses

by

Donna Yee

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Cell and Systems Biology
University of Toronto

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Abstract

The ability of plants to sense and respond to environmental and endogenous signals is essential to their growth and development. As part of these diverse cellular functions, ubiquitin-mediated proteolysis has emerged to be an important process involved in how plant signalling pathways can be regulated in response to such cues. Of the three enzymes involved in linking ubiquitin to protein targets, E3 ubiquitin ligases are of interest as they confer substrate specificity during this ubiquitination process. The overall focal point of this research is on plant U-box (PUB) E3 ubiquitin ligases, a family that has undergone a large gene expansion possibly attributable to the regulation of biological processes unique to the plant life cycle. In Arabidopsis there are 64 predicted PUBs, many for which biological roles have yet to be determined. And as research continues to uncover PUB functions, the functional diversity in the gene family will likely expand.

Specifically the focus of this research is on characterizing two ARM repeat-containing PUBs – AtPUB18 and AtPUB19. General analysis of pub18 and pub19 T-DNA insertion
lines for growth defects did not yield distinct altered phenotypes. Closer inspection of selected lines showed independent gene assortment phenotypes that, with further inordinately convoluted pursuit, proved to have an AtPUB18/19-unrelated outcome. The availability of Arabidopsis microarray databases provided exploratory expression profiling as a starting point to elucidate PUB function. AtPUB19 and closely related AtPUB18 are notable for their increased expression during abiotic stresses. While condition-directed germination assays showed a decreased sensitivity to salt and ABA for pub18 pub19 double insertion lines, no related change in susceptibility to these or other abiotic stress treatments were seen with condition-directed root growth assays. Thus, this preliminary work has begun to reveal insight into the complex abiotic stress-related roles AtPUB18 and AtPUB19 have during mediation of environmental stress acclimation in Arabidopsis.
ACKNOWLEDGEMENTS

After six years endeavouring in the pursuit of this degree, the list of people who have contributed in some sort of form – be it through scholarly advice and mentoring or morale-providing coffee and food breaks – and thus warrant a mention here has become quite extensive and I apologize if I’ve left you out. Know that I am very thankful to all of you that have offered your sincere and true support and help during this process in achieving this end result: the final dissertation.

I happily take this section to give special shout-outs to those people who have gone that extra mile in making my journey memorable.

Thank you Dr. D. Goring for your welcome into your lab. My experience during my years under your supervision would not have been the same without all that support and encouragement provided in show of just how much you believed in me.

Thank you to my advisory committee members, Drs. K. Yoshioka and N. Provart for your advice and guidance throughout the completion of my degree. Your patient and understanding faces have been a comfort at every committee meeting.

Special thanks go out to all present and past Goring lab members, affectionately known as the “Goring Girls”, for your camaraderie and so many life lessons learned through your words, laughter, and most of all, your actions. My apologies to you L.C. (alas, you know why) but you’re a better person for it and you will be a great teacher.

Having read, edited, formatted, assembled, etc. so many theses (four at last count, not including my own!) that have come from this lab, I know that the person who has taken so much time out of their days and nights by doing so deserves an extra acknowledgement: Gratitude goes to you K.H.! And that last sentiment needs to be extended to beyond your “sphell chequeng and grammer” skills. I know you want to have a grossly affectionate paragraph praising you, so the pressure is on to at least make it witty, even if the former is not quite the way I roll. I guess I can start by acknowledging you for being my secondary feeder (see below for primary). Apparently, we have hung out long enough for you to realize my dietary needs, although relatively
low maintenance, does not include greenery. Thank you for making the last few years of my degree more palatable than a salad made without croutons, bacon bits, cheese, or any of the “good stuff”. I am convinced I would be in a much darker place if it weren’t for your introduction to Starbucks, outlet-shopping, and, of course your most adorable fur-baby Jellybean (“Beans”) Victoria. Thanks for convincing her I’m an awesome Auntie! Indeed, who knew that a coupon-clipping, Coach-loving, conference travel-buddy kindred spirit was sitting in the next office all this time.

My academic experience wouldn’t be this rich if it weren’t for the continually amazing encouragement of my M.Sc. supervisor Dr. S. Regan. Your generous nature continues to astound me. For all the reassuring chats, academic advice, bolstering support, and genuine belief that success in academia was not impossible, I (as so many of your former students are) will be eternally grateful (even if you did ruin us by spoiling us so much during your tutelage).

And last but certainly not least, the enduring support of my (then) significant-other and (now) husband J.M., emotionally (yes, I get grumpy when I’m tired and hungry, two feelings that often cropped up when thesis-writing occurred) and financially (agreed, Ramen noodles are no longer worth the health hazard their cheapness affords). Thanks for leading by example with your virtuous qualities, but also for accepting me even when I just watch (often with skepticaly raised eyebrow) these same qualities and don’t always (refuse to?) follow.

As a fun note, the original idea was to record the vast quantities of junk food consumed in desperate search of comfort while writing this thesis. As the list grew (and grew and grew), I remembered how my arse followed that same pattern when I was writing my Masters thesis seven years ago. Vowing to resist a similar experience, although I still stuffed every consumable fatty item within arms reach into my mouth, I was pre-emptive enough to counter with approximately 20 BodyFlow classes (give or take a half dozen or so) a month. So appreciation goes out to GoodLife Fitness (and their excellent fitness instructors) for having (and teaching) such a wonderful combination class of Yoga, Tai-chi, and Pilates. I am thrilled to be now part of this GroupEx instructor team. Namaste.

June 2010
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>20SP</td>
<td>20S proteasome</td>
</tr>
<tr>
<td>26SP</td>
<td>26S proteasome</td>
</tr>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>ABI</td>
<td>ABA-insensitive</td>
</tr>
<tr>
<td>ABRC</td>
<td>Arabidopsis Biological Resource Center</td>
</tr>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylate</td>
</tr>
<tr>
<td>ACRE</td>
<td>Avr9/Cf-9 rapidly elicited</td>
</tr>
<tr>
<td>ACS</td>
<td>ACC synthase</td>
</tr>
<tr>
<td>AGI</td>
<td>Arabidopsis Genome Initiative</td>
</tr>
<tr>
<td>AIP</td>
<td>ABI3-interacting protein</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase-promoting complex</td>
</tr>
<tr>
<td>ARC</td>
<td>ARM repeat-containing</td>
</tr>
<tr>
<td>ARF</td>
<td>auxin response factor</td>
</tr>
<tr>
<td>ARM</td>
<td>Armadillo</td>
</tr>
<tr>
<td>ASK</td>
<td>Arabidopsis SKP-related</td>
</tr>
<tr>
<td>At</td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AUX/IAA</td>
<td>auxin/indole-3-acetic acid</td>
</tr>
<tr>
<td>Avr</td>
<td>avirulence</td>
</tr>
<tr>
<td>BAR</td>
<td>Bio-Array Resource</td>
</tr>
<tr>
<td>Bc</td>
<td>Botrytis cinerea</td>
</tr>
<tr>
<td>Bg</td>
<td>Bruguiera gymnorhiza</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>BL</td>
<td>brassinolide</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>Bn</td>
<td><em>Brassica napus</em></td>
</tr>
<tr>
<td>BOP</td>
<td>blade-on-petiole</td>
</tr>
<tr>
<td>BTB</td>
<td>bric-a-brac, tramtrack, and broad-complex</td>
</tr>
<tr>
<td>BY-2</td>
<td>Bright Yellow - 2</td>
</tr>
<tr>
<td>Ca</td>
<td><em>Capsicum annuum</em></td>
</tr>
<tr>
<td>CAN</td>
<td>L-canavanine</td>
</tr>
<tr>
<td>CAO</td>
<td>chlorophyllide a oxygenase</td>
</tr>
<tr>
<td>CBF</td>
<td>core binding factor</td>
</tr>
<tr>
<td>CCS</td>
<td>cell cycle switch</td>
</tr>
<tr>
<td>CDC</td>
<td>cell division cycle protein</td>
</tr>
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<td>CDH</td>
<td>CDC-homology</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>Cf</td>
<td><em>Cladosporium fulvum</em>-derived</td>
</tr>
<tr>
<td>CHIP</td>
<td>carboxyl terminus of HSC70-interacting protein</td>
</tr>
<tr>
<td>Clp</td>
<td>chloroplast protease</td>
</tr>
<tr>
<td>CMPG</td>
<td>Cys, Met, Pro, Gly</td>
</tr>
<tr>
<td>COI</td>
<td>coronatine insensitive</td>
</tr>
<tr>
<td>Col</td>
<td>Columbia</td>
</tr>
<tr>
<td>CP</td>
<td>core particle</td>
</tr>
<tr>
<td>CRL</td>
<td>cullin-RING ligase</td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyl-trimethyl-ammonium bromide</td>
</tr>
<tr>
<td>C-term</td>
<td>carboxyl-terminus</td>
</tr>
<tr>
<td>CUL</td>
<td>Cullin</td>
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</table>
Cvi  Cape Verde Island
DDB  DNA-damage-binding
ddH₂O  distilled deionized water
DEPC  diethyl pyrocarbonate
DEX  dexamethasone
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
DRE  dehydration-responsive element
DREB  DRE-binding
DRIP  DREB2A-interacting protein
DSB  double-strand break
DUB  deubiquitinating enzyme
DWD  DDB1-binding WD40
E1s  E1 ubiquitin-activating enzymes
E2s  E2 ubiquitin-conjugating enzymes
E3s  E3 ubiquitin ligase enzymes
EBF  EIN3 binding F-box
EDTA  ethylenediaminetetraacetic acid
EIN  ethylene insensitive
ETI  effector-triggered immunity
ETO  ethylene overproducer
F1  first cross / first filial
F2  second filial
F-box  cyclin F protein
FBL  F-box like
FLARE flagellin rapidly elicited
flg flagellin
FLU fluridone
Fts thermosensitive filamentation
GA gibberellic acid / gibberellin
GABI-Kat Genomanalyse im biologischen system pflanze (Genome analysis in the plant biological system) - Kölner Arabidopsis T-DNA lines
gDNA genomic DNA
GID gibberellin insensitive dwarf
GR glucocorticoid receptor
GST glutathione S-transferase
GUS β-glucuronidase
HBD hormone-binding domain
HECT homology to E6-AP carboxyl terminus
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOS high expression of osmotically responsive genes
HR hypersensitive response
HrpZ hairpin Z
HSC heat shock cognate protein
HSP heat shock protein
IAA indole-3-acetic acid
ICE inducer of CBF expression
JA jasmonic acid / jasmonate
JAZ JA-ZIM domain
KEG keep on going
<table>
<thead>
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<th>Description</th>
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<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAC</td>
<td>MOS4-associated complex</td>
</tr>
<tr>
<td>MAMP</td>
<td>microbial-associated molecular pattern</td>
</tr>
<tr>
<td>MAX</td>
<td>more axillary growth</td>
</tr>
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<td>MES</td>
<td>4-morpholine-ethanesulfonic acid</td>
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<td>PCD</td>
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<td>PHOR</td>
<td>photoperiod responsive</td>
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<td>pox virus and zinc finger</td>
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<td><em>Pseudomonas syringae</em> pv. <em>tomato</em></td>
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<td>PAMP-triggered immunity</td>
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<td>shoot apical meristem</td>
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<tr>
<td>SAR</td>
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CHAPTER 1: LITERATURE REVIEW

My thesis focuses on determining what role two selected Arabidopsis U-box N-terminal domain (UND)-containing AtPUB-ARMs – AtPUB18 and AtPUB19 – may have during plant growth and development. In order to put my research into context, a background highlighting relevant topics will first be presented. The brief introduction will include details behind the ubiquitination process, the apparatus and purpose behind this post-translational modification, and lastly, information about the proteasome, the most common destination of ubiquitinated substrates. Among the enzymes involved in ubiquitination, the E3 ubiquitin ligases (E3s) and their classification will be covered, specifically in plants. In particular, focus will be given to the U-box family of E3 ubiquitin ligases and to examples of their involvement in a diversity of functions. Research on PUB genes from several different plants, highlighted primarily in Arabidopsis thaliana, will be presented to illustrate the range of functions for this gene family. Connections between PUB genes and hormonal, abiotic stress, and biotic defence responses will be discussed. To facilitate future understanding of these connections, background information is given on ABA as an influential hormone during germination and abiotic stress signalling, on salt, drought, and cold as similar abiotic stress signals during plant adjustment to environmental changes, and on pathogen invasion as a trigger for plants to regulate defensive immunity. Finally, how programmed cell death and its outcome from abiotic and biotic ABA stress signalling may be interconnected to ubiquitination and proteasome-mediated protein degradation will be reviewed as a possible encompassing theme underlying general PUB function.

The following introduction contains excerpts from a review “The diversity of plant U-box E3 ubiquitin ligases: from upstream activators to downstream target substrates” recently published in the Journal of Experimental Botany (Yee and Goring, 2009).

1 Introduction

With the reliance of plant growth and development on the ability of plants to sense and respond to many environmental and endogenous signals, the network of interactions required to coordinate the appropriate response to stimuli perception is expectedly
complex. Amongst the intricacy, besides the obvious necessity of transmission of the signal, is the regulation of signal transduction pathways themselves. What has significantly emerged as an important post-translational process during the modulation of eukaryotic signalling pathways responding to developmental cues or to acclimate to environmental stress has been ubiquitination-mediated proteolysis. This modification process has gained even further attention in plants because of the considerable increase in ubiquitin-proteasome system-related genes that have been identified in several plant genomes (reviewed in Smalle and Vierstra, 2004; Stone and Callis, 2007).

1.1 Ubiquitination

The act of ubiquitination (also known as ubiquitylation) involves the attachment of ubiquitin moieties to target proteins (Figure 1). The multi-step selective ligation of ubiquitin (Ub), a highly conserved polypeptide of 76 amino acids (Wilkinson et al., 1980; Vijay-Kumar et al., 1987), itself involves a cascade mediated by three sequential ubiquitination enzymes. The molecule is activated through an ATP-dependent formation of thiol-ester linkage between the ubiquitin's C-terminus (glycine residue) and the reactive site (cysteine residue) in an E1 ubiquitin-activating enzyme. The thiol-ester-linked ubiquitin is then transferred to the active site of an E2 ubiquitin-conjugating enzyme. Next, an E3 ubiquitin-ligase enzyme interacts with both the ubiquitin-carrying E2 enzyme and a specific protein to mediate, directly (E2-to-E3-to-substrate) or indirectly (E2-to-substrate), the transfer of the activated ubiquitin to the targeted protein. The ubiquitin is linked through a stable isopeptide bond between a C-terminal glycine residue in ubiquitin and a lysine residue in the target. Multiple rounds of conjugation can then add more ubiquitin units to the target protein (reviewed in Kurepa and Smalle, 2008a; Smalle and Vierstra, 2004).

The fate of ubiquitinated proteins depends on several factors, including how many ubiquitins are attached and where the ubiquitination occurs. With the attachment of unlinked single ubiquitin moieties (either as a sole molecule or multiple lone molecules along the protein length), these monoubiquitination events have been associated with modification of the target protein’s localization, activity or interactions (Hicke, 2001; Schnell and Hicke, 2003). As ubiquitin also carries its own internal lysine residues
Figure 1: An overview of the ubiquitination cascade and the proteolytic degradatory pathway.

The act of ubiquitination involves the attachment of ubiquitin moieties to target substrates. The multi-step selective ligation of ubiquitin (Ub), a highly conserved polypeptide of 76 amino acids, involves a cascade mediated by three sequential ubiquitination enzymes. The molecule is activated through an ATP-dependent formation of thiol-ester linkage between ubiquitin and an E1 ubiquitin-activating enzyme. The activated ubiquitin is then transferred to the E2 ubiquitin-conjugating enzyme. Next, an E3 ubiquitin-ligase enzyme interacts with both the ubiquitin-carrying E2 enzyme and a specific protein substrate to mediate, directly (E2-to-E3-to-substrate) or indirectly (E2-to-substrate), the transfer of the activated ubiquitin to the targeted substrate. With the attachment of unlinked single ubiquitin moieties, these monoubiquitination events have been associated with modification of the target protein’s localization, activity or interactions.

Multiple rounds of conjugation can add more ubiquitin units to the target protein, yielding chains of polyubiquitin that lead to their escort to the 26S proteolytic degradatory pathway. At the 26S proteasome, the 19S regulatory particles capping both cylindrical ends of the 20S proteolytic core particle function to recruit polyubiquitinated proteins. This recruitment would entail recognition of polyubiquitin chains, capture of such ubiquitinated proteins, release of reusable ubiquitin moieties from degradation-bound substrates, unfolding of these substrates, followed by their guided entry into the CP proteolytic core, where the internal chamber housing the active sites for proteolysis would degrade proteins directed into this central core. Ubiquitinated target proteins endure degradation into small peptides and, along with recycled ubiquitin moieties, are all subsequently released by the 26S proteasome and scavengeable for reuse by the cell.
Ubiquitin + ATP → ADP + PPI

Ubiquitin Activation

Ubiquitin Conjugation

Ubiquitin Ligation

Monoubiquitination

Polyubiquitination

Substrate Degradation

Recognition
Deubiquitination
Unfolding
Internalization

19S regulatory particle
20S proteolytic core
26S proteasome

Substrate Modification
(ex. localization, activity, interactions)
(seven; Pickart and Fushman, 2004), subsequent rounds of ubiquitination can also add upon an existing ubiquitin moiety to yield a target with chains of polyubiquitin. Typically, polyubiquitination occurs at ubiquitin’s Lys-48, but non-canonically-linked reversible multi-ubiquitin chains at other lysines (Arnason and Ellison, 1994; Baboshina and Haas, 1996) have been associated with non-proteolytic cellular functions like kinase activation, vesicular trafficking, DNA repair, and endocytosis (Bach and Ostendorff, 2003; Pickart and Fushman, 2004; Mukhopadhyay and Riezman, 2007). The best known and best characterized consequence of Lys-48-linked polyubiquitinated proteins, however, is their breakdown through the 26S proteolytic degradatory pathway.

1.2 26S proteasome

As the last step in the ubiquitin-26S proteasome system (UPS), polyubiquitinated target proteins are escorted to the 26S proteasome (26SP), a cytoplasm- and nuclear-located ATP-dependent multi-subunit protease of 2.5MDa responsible for selective protein degradation (Smalle and Vierstra, 2004; Yang et al., 2004). This pathway provides important cellular quality-control/housekeeping duties through its elimination of non-functional proteins made aberrant by mistranslation, truncation, or misfolded conformations, but the additional degradation mediated by this system has also emerged as a significant means to regulate protein activity by providing an effective counterbalance to protein synthesis (Smalle and Vierstra, 2004). Upon being accepted into the UPS, ubiquitinated target proteins endure degradation into small peptides and recycled ubiquitin moieties that are all subsequently released by the 26S proteasome and scavengeable for reuse by the cell. The 26S proteasome itself is a large complex composed of a central 20S proteolytic core particle (CP) flanked by two cap-like 19S regulatory particles (RP) each with lid and base substructures (Voges et al., 1999; Smalle and Vierstra, 2004).

1.2.1 20S proteolytic core

The protease activity of the 26S proteasome is driven by the 20S proteolytic core particle. In the CP, which is assembled by four heptameric rings stacked to form a hollow barrel-like cylinder, the internal chamber houses the active sites for proteolysis and proteins directed into this central core are degraded (Voges et al., 1999; Smalle
and Vierstra, 2004). The proteolytic core itself can exist in a free-form capable of mediating ubiquitin-independent proteolysis (Asher et al., 2006) and as such is referred to as the 20S proteasome (20SP). Even devoid of regulatory particles, the opening into the protease-active internal chamber of free 20SP is not so permissive as to allow random substrate entry (Groll et al., 2000), and thus protein degradation by this form of CP is still deliberately restricted.

1.2.2 19S regulatory particle

When 19S regulatory particles cap both cylindrical ends of the 20S core particle to generate the 26S proteasome, degradation is directed towards ubiquitin-dependency (Smalle and Vierstra, 2004). As a collective unit, the lid and base subcomplexes of each 19S RP function to recruit polyubiquitinated proteins into the site of proteolysis (20S CP). More specifically, this recruitment would entail recognition of polyubiquitin chains, capture of such ubiquitinated proteins, release of reusable ubiquitin moieties from degradation-bound substrates, unfolding of these substrates, followed by their guided entry into the CP proteolytic core (Vierstra, 2009). While the RP base comprises a ring of triple A (AAA⁻)->ATPase subunits (RPT1-6) and non-ATPase subunits (RPN1, RPN2, RPN10), the RP lid consists of RPN3, RPN5-9, RPN11-12 (Smalle and Vierstra, 2004). Each subunit presumably has a function related to the purpose of the regulatory particle and so far, RPN10 and RPN13 have been identified as potential ubiquitin receptors (Deveraux et al., 1994; Hiyama et al., 1999; Husnjak et al., 2008), RPN11 as a deubiquitinating enzyme (DUB) required for bound-ubiquitin chain release (Verma et al., 2002; Yao and Cohen, 2002), and RPTs as the energy-utilizing subunits involved in protein unfolding and/or unfolded protein allowance into the interior chamber of the CP (Groll et al., 2000), although such specific functions have yet to be attributed for each RP subunit.

1.3 E3 ubiquitin ligases as directors of ubiquitination specificity

The abundance of proteins with sequence homology to known components of the ubiquitin-proteasome pathway in the predicted Arabidopsis thaliana proteome illustrates how important this selective protein degradation system is in plants. It has been postulated that more than 6% (>1600 genes) of the Arabidopsis genome encodes main
components that function in the UPS (Vierstra, 2009). These include sixteen ubiquitin genes, two E1 activating enzyme genes, at least forty-five E2 and E2-like conjugating enzyme genes, twenty-three 20S core protease genes, and thirty-one 19S regulatory particle genes, along with other factors such as deubiquitinating enzymes, with the remaining >1400 genes encoding E3 ubiquitin ligase components (reviewed in Smalle and Vierstra, 2004; Vierstra, 2009).

The large number of E3 ubiquitin ligase genes relative to other Ub pathway-related genes in Arabidopsis and other eukaryotes is indicative of the importance of the E3 ubiquitin ligase step during the tightly directed selectivity of the ubiquitin-proteasome pathway. As the enzyme that interacts with the targeted protein, the diverse E3 ubiquitin ligases in eukaryotes are considered to be the factors conferring specificity to the ubiquitination process. As a consequence, these proteins are of high interest to researchers interested in studying the regulatory roles of E3 ubiquitin ligases during organism growth and development.

What distinguishes plant E3 ubiquitin ligases from other eukaryotes E3 ubiquitin ligases is the expansion in their numbers (Patterson, 2002). In fact, many of the gene families governing the main components of the plant UPS are expanded in comparison to their human, mice, Drosophila, and yeast counterparts (Smalle and Vierstra, 2004; Vierstra, 2009). The diversity and complexity of the plant UPS in conjunction with the evolution of so many different plant E3 ubiquitin ligase components implies that the plant proteome may be an enormous source of potential protein substrates to be recognized and targeted for unique ubiquitination cascades. Thus, while regulated protein degradation has clearly emerged to be an important developmental step during the establishment and maintenance of eukaryotic form and function, the high number of E3 ubiquitin ligases in plants suggests that in plants the use of protein turnover as means to actively and precisely modulate the complex interactions behind plant responses during growth and development and acclimation is more prevalent.

The diversity of the E3 ubiquitin ligases can be divided into various families based on their mechanism of action and on the presence of other specific HECT, RING, or U-box domains (Mazzucotelli et al., 2006).
1.3.1 **HECT ligases in plants**

HECT-type E3 ubiquitin ligases were originally identified because they possess a consensus domain with homology to the carboxyl terminus of the prototype human E6-AP ubiquitin ligase (HECT) (Huibregtse et al., 1995). These single subunit E3 ubiquitin ligases consist of a typically >100kDa polypeptide that, through the C-terminal HECT domain, interacts with the Ub-conjugated E2 to form an intermediate Ub-E3 linkage before the ubiquitin is stably transferred to the target substrate that is predicted to interact with protein-interaction motifs in the region upstream of the HECT domain (Sullivan et al., 2003). There are 7 HECT-type predicted E3 ubiquitin ligases in Arabidopsis and the variety of upstream interaction domain suggests that each function on a distinct set of targets (Bates and Vierstra, 1999; Downes et al., 2003).

1.3.2 **RING ligases in plants**

RING-type (Really Interesting New Gene) E3 ubiquitin ligases were originally identified from the similarity of their conserved RING domain to the hallmark zinc-binding, cysteine- and histidine-rich motif found in the prototype RING-finger module (Lovering et al., 1993). These E3 ubiquitin ligases can be sub-classified into simple single-subunit E3 ubiquitin ligases or complex multi-subunit E3 ubiquitin ligases, the latter of which includes the RING- and CULLIN-based complexes and the anaphase-promoting complex (APC) ligases (Mazzucotelli et al., 2006). Regardless of the number of subunits present, the RING E3 ubiquitin ligases never form a direct linkage to the ubiquitin moiety but instead provide the scaffolding to bind the Ub-conjugated E2, through the RING domain, and to orientate the E2 towards the target protein during ubiquitin transfer (Jackson et al., 2000). The Arabidopsis genome contains 469 RING proteins (Stone et al., 2005) with the potential of 30 more active E3 ubiquitin ligases (Mazzucotelli et al., 2006).

As a multi-subunit complex, cullin-RING ligases (CRLs) use RING and cullin proteins as the founding subunits, and variable factors as additional adapter components; the former of which binds the E2-ubiquitin-conjugating enzyme, the latter of which forms the substrate recognition module. In the canonical SCF-type (SKP1-CUL1-F-box) E3 ubiquitin ligases, the four-subunit E3 ubiquitin ligase complex is characterized by a
RING-box protein (RBX), an S-phase kinase associated protein (SKP), a CULLIN1 (CUL1), and an F-box protein. The cullin-bound RBX confers the catalytic E3 ubiquitin ligase activity, and the scaffold-like cullin binds with a linker SKP, or ASK (Arabidopsis SKP), which interacts with the N-terminal F-box motif found in F-box subunits, the latter of whose other protein-protein interaction domains are presumed to confer substrate specificity to the whole E3 complex (Zheng et al., 2002). By changing the cullin bridge between the catalytic module (E2-recruiting RBX1) and the adapter module (substrate-recognition components), the ubiquitination of substrate targets can be varied and other SCF-like CRL families generated. Thus by changing the scaffold to CUL3, dual function proteins with a Bric-a-brac, Tramtrack, and Broad-complex (BTB) / Pox virus and Zinc finger (POZ) motif interact with CUL3 through this domain and with targets through additional protein-protein interaction domains (Geyer et al., 2003; Xu et al., 2003; Pintard et al., 2004). The CUL4 scaffold associates with linker UV-DNA damage-binding (DDB) proteins, which in turn interact with substrate-recruiting DDB1-binding WD40 (DWD) proteins through the DWD motif, the latter proteins mediating target recognition (He et al., 2006; Lee et al., 2008). APC complexes are the most elaborate, containing 11 or more subunits, including cullin-related (APC2) and RBX1-related (APC11) as the minimal core, and interchangeable substrate-recognition subunits like WD40 proteins Cell Division Cycle protein 20 (CDC20) and CDC-Homology 1 (CDH1), known as Cell Cycle Switch 52 (CCS52) in plants, with the other subunits, like CDC27, presumably mediating APC complex interactions (Capron et al., 2003a; Capron et al., 2003b; Vodermaier et al., 2003; Fulop et al., 2005). The possible structural combinations between the 2 RBXs, 6 cullins, 21 ASKs, 694-703 F-box proteins, 81 BTBs, 2 DDBs, 85 DWDs, 6 CDC20s, 3 CCS52s, 2 CDC27s predicted from the Arabidopsis genome (Gagne et al., 2002; Risseeuw et al., 2003; Capron et al., 2003a; Dieterle et al., 2005; Fulop et al., 2005; Gingerich et al., 2005; Mazzucotelli et al., 2006), means the large number of putative SCFs, cullin-BTBs, cullin-DDBs, and APCs may have significant implications in our understanding of plant development.

1.3.3 U-box ligases in plants

As the most recently discovered class of E3 ubiquitin ligases, U-box E3 ubiquitin ligases are categorized based on a conserved ~70 amino acid U-box motif originally identified
in the yeast Ub Fusion Degradation 2 (UFD2) protein, a ubiquitin chain elongation factor (Koegl et al., 1999; Hatakeyama and Nakayama, 2003). Sequence and predicted secondary structure analyses revealed that the U-box was a modified RING-finger domain, despite the lack of the scaffold-stabilizing, zinc-chelating cysteine and histidine residues characteristically conserved in the RING domain (Aravind and Koonin, 2000). Other intramolecular interactions, such as salt bridges and hydrogen bonds, are proposed to replace the RING zinc-cysteine-histidine coordination to stabilize the U-box scaffold (Aravind and Koonin, 2000). U-box proteins do function as E3 ubiquitin ligases and indirectly mediate ubiquitin ligation by simultaneously docking both the Ub-loaded E2 enzymes and the targeted substrates (Jackson et al., 2000; Hatakeyama et al., 2001).

1.4 U-box E3 ubiquitin ligases – a gene expansion in plants

Unlike the RING and HECT domain E3 ubiquitin ligases, where the numbers of these predicted genes are relatively the same among eukaryotic species, a significantly larger number of U-box containing genes are predicted in various plant genomes (Patterson, 2002). In comparison to the 2 and 21 U-box genes identified in the yeast and humans genomes, respectively (Koegl et al., 1999; Hatakayama et al., 2001; Ohi et al., 2003), 64 U-box genes have been predicted in the Arabidopsis genome (Azevedo et al., 2001; Wiborg et al., 2008), and 77 have been annotated in the rice genome (Zeng et al., 2008). This evolution of expanded plant U-box (PUB) proteins may indicate not only their importance in governing cellular processes that are specific to plants, but also the wide range of functional involvement these proteins could have as part of regulated plant growth and development.

The U-box containing proteins found in plants can be further classified on the basis of the presence of other domains (Azevedo et al., 2001; Andersen et al., 2004; Mudgil et al., 2004; Samuel et al., 2006; Wiborg et al., 2008). At 64%, the largest class of plant U-box proteins in Arabidopsis is the Armadillo (ARM) repeat domain-containing PUB proteins (Mudgil et al., 2004; Samuel et al., 2006; Wiborg et al., 2008). At 64%, the largest class of plant U-box proteins in Arabidopsis is the Armadillo (ARM) repeat domain-containing PUB proteins (Mudgil et al., 2004; Samuel et al., 2006; Wiborg et al., 2008). At 64%, the largest class of plant U-box proteins in Arabidopsis is the Armadillo (ARM) repeat domain-containing PUB proteins (Mudgil et al., 2004; Samuel et al., 2006; Wiborg et al., 2008). At 64%, the largest class of plant U-box proteins in Arabidopsis is the Armadillo (ARM) repeat domain-containing PUB proteins (Mudgil et al., 2004; Samuel et al., 2006; Wiborg et al., 2008). At 64%, the largest class of plant U-box proteins in Arabidopsis is the Armadillo (ARM) repeat domain-containing PUB proteins (Mudgil et al., 2004; Samuel et al., 2006; Wiborg et al., 2008). At 64%, the largest class of plant U-box proteins in Arabidopsis is the Armadillo (ARM) repeat domain-containing PUB proteins (Mudgil et al., 2004; Samuel et al., 2006; Wiborg et al., 2008). At 64%, the largest class of plant U-box proteins in Arabidopsis is the Armadillo (ARM) repeat domain-containing PUB proteins (Mudgil et al., 2004; Samuel et al., 2006; Wiborg et al., 2008). At 64%, the largest class of plant U-box proteins in Arabidopsis is the Armadillo (ARM) repeat domain-containing PUB proteins (Mudgil et al., 2004; Samuel et al., 2006; Wiborg et al., 2008). At 64%, the largest class of plant U-box proteins in Arabidopsis is the Armadillo (ARM) repeat domain-containing PUB proteins (Mudgil et al., 2004; Samuel et al., 2006; Wiborg et al., 2008). At 64%, the largest class of plant U-box proteins in Arabidopsis is the Armadillo (ARM) repeat domain-containing PUB proteins (Mudgil et al., 2004; Samuel et al., 2006; Wiborg et al., 2008).
located, tandemly-repeated ARM motifs (Samuel et al., 2006). Each ARM motif is approximately 42 amino acids long, and individually is non-functional, but when tandemly arranged, they comprise the ARM repeat domain (Groves and Barford, 1999). Despite each individual ARM motif having significant sequence divergence, there is an overall conserved tri-helical secondary structure, and all ARM motifs collectively together in the repeat domain is generally accepted to be needed for folding into the superhelical structure that is predicted to form a specific protein interaction domain (Huber et al., 1997). Interestingly, when a search of ARM repeats associated with other conserved domains was performed in other eukaryotic organisms, the largest family was the PUB-ARM family specific to Arabidopsis and rice, suggesting that the evolution of expanded U-box proteins in plants may be courtesy of expanding the PUB-ARM combination (Samuel et al., 2006).

At 23%, the next significant classification of Arabidopsis PUB proteins is the kinase domain-containing members (Wiborg et al., 2008). Smaller sub-families are represented by other predicted domains such as WD40 repeats and the MIF4G motif. AtPUB49 is the sole PUB gene in Arabidopsis with a C-terminal cyclophilin-like domain and belongs to the 29-member molecular chaperone Cyp protein family in Arabidopsis (Cyp65; He et al., 2004). This domain has been implicated in conferring the PPIase (peptidyl-prolyl cis-trans isomerase) activity that is needed for proper protein folding (Barik, 2006). AtPUB49’s cis-to-trans isomerisation of proline peptide bonds (and chaperone activity) has been determined in vitro (Wiborg et al., 2008). AtCHIP is the only plant U-box protein with a biological role described to date that is not a PUB-ARM protein and is characterized by the presence of three tandem tetratricopeptide repeats (TPR) (Yan et al., 2003). Like the ARM repeat domain, the TPR repeats are part of the large class of helical repeat domains (Groves and Barford, 1999) are also thought to be involved in protein-protein interactions (Blatch and Lassle, 1999).

The conserved nature of the U-box domain has allowed for easy searches within an individual protein sequence or entire sequence databases to identify proteins that can be classified under this U-box E3 ubiquitin ligase family. And the commercial availability of E1 and E2 enzymes and ubiquitin has allowed the assessment of the E3 ubiquitin ligase activity for any putative U-box protein using an in vitro ubiquitination assay. In
relative comparison to the identification and confirmation of plant U-boxes as E3 ubiquitin ligases, the next relevant step of elucidating their biological function is much more complex: whether an alteration in phenotype can be derived from loss of function and/or gain of function mutations or whether a phenotypic mutant of interest can be mapped back to a U-box containing protein. Finding the regulatory roles associated with PUB protein functions has revealed the importance of this directed-ubiquitination and protein degradation in many interesting processes in plants.

1.5 PUB proteins in growth and development

Because plant growth and developmental processes are so closely intertwined with hormone signalling, the list of E3 ubiquitin ligase components associated solely with morphological development is not as extensive (Stone and Callis, 2007), and in fact, PUB E3 ubiquitin ligases have yet to be directly associated with plant morphogenesis regulation without hormonal influences (Yee and Goring, 2009). Given the importance of successful plant reproduction to further plant development, plant responses during self-incompatibility and pseudo-self-compatibility, although technically a type of biotic response, are the closest regulatory roles PUB proteins hold during plant growth and developmental processes that have emerged so far.

The first PUB protein to be functionally characterized in plants was *Brassica napus* Arm Repeat-Containing 1 (ARC1), a member of the PUB-ARM family. Extensive work has characterized ARC1 to have E3 ubiquitin ligase activity that acts as a positive regulator of the *Brassica* self-incompatibility response (Stone et al., 1999, 2003). The first evidence for such a role was uncovered through the antisense suppression of *ARC1* in the stigma which resulted in self-incompatible pollen adhering and germinating to allow pollen tube penetration through the stigma (Stone et al., 1999). In this system, the signal initiating the response is the allele-specific male component, the SP11/SCR pollen coat protein (Schopfer et al., 1999; Takayama et al., 2000), binding to the female component, the plasma membrane-bound S Receptor Kinase (SRK), found in the stigmatic papillae (Kachroo et al., 2001; Takayama et al., 2001). SRK activation initiates the signalling cascade, along with the M Locus Protein Kinase, to prevent fertilization by any self-pollen (Takasaki et al., 2000; Silva et al., 2001; Murase et al., 2001).
As part of this cascade, ARC1 binds to SRK through the former’s ARM repeat domain and functions downstream of the activated SRK (Gu et al., 1998; Stone et al., 1999, 2003). The involvement of ubiquitin-mediated proteolysis in this response was confirmed with the U-box dependent co-localization of ARC1 with subunits of the proteasome, the increase in ubiquitinated proteins in the pistil following self-incompatible pollinations, and the breakdown of self-incompatibility upon treatment with proteasomal inhibitors (Stone et al., 2003).

The Arabidopsis thaliana PUB protein with the highest sequence homology to BnARC1 is AtPUB17. However, AtPUB17 does not appear to be a functional ortholog, given that Arabidopsis thaliana is a self-compatible plant and that AtPUB17 is much more broadly expressed (BnARC1 is exclusively expressed in the stigma; Gu et al., 1998). As well, Arabidopsis thaliana plants with AtPUB17 knocked out have shown phenotypes that are unrelated to plant reproduction (described in more detail below; Yang et al., 2006). Nonetheless, a self-incompatibility response has been induced in Arabidopsis thaliana with the introduction of self-incompatible Arabidopsis lyrata SCR and SRK genes, though the response can be transient when these genes are introduced into different Arabidopsis thaliana ecotypes (Nasrallah et al., 2002, 2004). Interestingly, one of the genes implicated in this pseudo self-compatibility phenomenon is AtPUB8 where a correlation was found between increased AtPUB8 expression and increased SRK mRNA levels, implicating AtPUB8 as a regulator of SRK transcript levels (Liu et al., 2007).

1.5.1 Other E3 ubiquitin ligases in plant growth and development

In Arabidopsis, several other E3 ubiquitin ligases types have been connected to aspects of plant development. So far, one member of the HECT-containing ubiquitin-protein ligase (UPL) family has been implicated in proper leaf trichome morphogenesis (UPL3; Downes et al., 2003). Amongst the RING-containing single E3 ubiquitin ligase components, BIG BROTHER and Shoot Apical Meristem Arrest (SHA) 1 have been identified as organ size and apical meristem regulators, respectively (Disch et al., 2006; Sonoda et al., 2007). Meanwhile, several F-box E3 ubiquitin ligases and a BTB-based E3 ubiquitin ligase, as part of the multi-subunit E3 ubiquitin ligase complexes, are
involved in plant growth and development without yet any specific linkage to hormonal pathways; Unusual Floral Organs (UFO) is important for patterning in flowers (Samach et al., 1999; Hepworth et al., 2006), Vier F-box Proteins (VFB) 1-4 for lateral root growth (Schwager et al., 2007), F-box Like (FBL) 17 for production of sperm cells during pollen development (Kim et al., 2008; Gusti et al., 2009), and Blade-On-Petiole (BOP) 1 and BOP2 for initiation of lateral organs and abscission (Hepworth et al., 2005; McKim et al., 2008).

1.6 PUB proteins in hormonal responses

Similar to the discovery of ARC1, the potato (Solanum tuberosum ssp. andigena) Photoperiod Responsive 1 (PHOR1) was identified through its positive regulatory role during gibberellic acid (GA) signalling (Amador et al., 2001) before being confirmed as a U-box protein (Monte et al., 2003). PHOR1 was originally identified through its photoperiod-dependent up-regulation, and antisense suppression of PHOR1 produced semi-dwarf plants with higher endogenous GA levels and decreased sensitivity to low levels of exogenous GA, all phenotypes being indicative of impaired GA responsiveness (Amador et al., 2001). Conversely, PHOR1-over-expressing lines showed increased sensitivity to exogenous GA applications and increased resistance to the effects of paclobutrazol, a GA biosynthesis inhibitor, during internode elongation, both indicative of enhanced GA responsiveness (Amador et al., 2001). Having shown the various effects on GA responses being mediated through the down-regulation and up-regulation of PHOR1, it will be interesting to see if AtPUB27, AtPUB28, and AtPUB29 (HIM3, HIM1, HIM2), the Arabidopsis PUB proteins most similar to StPHOR1, will have similar roles in GA responses in Arabidopsis (Monte et al., 2003; Thomas and Sun, 2004).

With the role of PHOR1 during GA responses in potato, the question arises as whether other PUB proteins are involved in phytohormone responses. The observation that several plant hormones stimulate the expression of a number of AtPUB genes (Figure 2A) serves as a suitable starting point in determining whether any other hormone-related regulation by PUB proteins might be occurring. For example, the AtPUB9 gene is responsive to ABA treatment (Figure 2A), and using this information, a connection was subsequently established between AtPUB9 and ABA responses (Samuel et al.,
Figure 2: Gene expression microarray profiles for PUB genes in Arabidopsis from the AtGenExpress Consortium data sets under a range of conditions and treatments.

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A) AtPUB expression profiles in response to hormone treatment in wild-type seedlings and seeds. Seedlings samples are shown (each block a time point from left to right) at 0.5, 1, and 3 hours of hormone treatment with 1μM of Zeatin, IAA or gibberellin3 (GA3); or 10μM 1-aminocyclopropane-1-carboxylate (ACC), methyl jasmonate (MJa), abscisic acid (ABA), or brassinolide (BL). Seed samples are shown at 24 hours following 3μM or 30μM ABA treatment (Goda et al., 2008).

B & C) AtPUB expression profiles in response to various abiotic stress treatments on wild-type seedlings in the shoots (B) and roots (C). For the cold, osmotic, and salt treatments, samples are shown (each block a time point from left to right) at 0.5, 1, 3, 6, 12, and 24 hours. For drought, UV-B and wounding treatments, samples are shown at 0.25, 0.5, 1, 3, 6, 12, and 24 hours. For heat treatment, samples are shown (each block a time point from left to right) at 0.25, 0.5, 1, 3, 4, 6, 12 and 24 hours (Killian et al., 2007).

D) AtPUB expression profiles in response to pathogen infections or elicitor treatments. Pseudomonas syringae pv. tomato DC3000 (Pto DC3000) and Pseudomonas syringae pv. phaseolicola (Psp) infections are shown (each block a time point from left to right) at 2, 6, and 24 hours. Elicitor treatments are shown (each block a time point/treatment from left to right) for the following treatments (at 1 and 4 hours): flagellin22 (Flg22), glutathione S-transferase (GST) control, GST-Nucleotide Pyrophosphatase/Phosphodiesterase1 (NPP1), hairpin Z (HrpZ), lipopolysaccharide (LPS), and magnesium chloride (MgCl) control. Phytophthora infestans (Pi) infections are shown (each block a time point from left to right) at 6, 12, and 24 hours. Botrytis cinerea (Bc) infections are shown (each block a time point from left to right) at 18 and 48 hours.
Heat maps from the *AtGenExpress Consortium* data sets (Killian *et al*., 2007; Goda *et al*., 2008) were generated at the Bio-Array Resource website (bar.utoronto.ca; Toufighi *et al*., 2005). All 64 presently known *AtPUB* genes were queried, however only the *AtPUB* genes with increased expression profiles under the selected conditions are shown. *AtPUB4, AtPUB31, AtPUB38, AtPUB39, AtPUB41, AtPUB46, AtPUB47, AtPUB54, AtPUB61 and AtPUB62* were not present on the microarrays. For the gene identifiers (AGI numbers) associated with the AtPUB protein names, please refer to www.arabidopsis.org and Wiborg *et al*. (2008) or Appendix IV.
AtPUB9 was found to relocalize from the nucleus to the plasma membrane with ABA treatment, following transient expression in tobacco BY-2 cells. As well, *Arabidopsis pub9* knock-out lines showed hypersensitivity to ABA during seed germination assays. Crosses between the ABA-insensitive *abi3*-6 mutant and the *pub9* mutant found that the *abi3*-6 phenotype was epistatic to *pub9* in seed germination assays (Samuel et al., 2008).

### 1.6.1 Other E3 ubiquitin ligases in hormonal responses

Given the magnitude of influence hormone signalling has throughout growth and development during a plant’s entire lifetime, it is not surprising that the UPS plays a significant role in hormonal responses. When E3 ubiquitin ligases are considered as the UPS components of focus, their regulatory control has been noted in many aspects of hormones, from biosynthesis, perception, to downstream signalling to effect responses (Stone and Callis, 2007).

In view of hormone biosynthetic pathways in *Arabidopsis*, the exemplary E3 protein is the Ethylene Overproducer (ETO) 1 during ethylene synthesis. By modulating the rate-determining step of a biosynthetic pathway, an E3 ubiquitin ligase could effectively govern hormone synthesis. ACC synthases (ACSs) are such a biosynthetic control point and their interaction (specifically ACS5, a type 2 ACS) with E3 ubiquitin ligases (specifically ETO, a BTB-assembled multiunit E3 ubiquitin ligase) and subsequent destabilization is correlated with decreased ethylene production (Wang et al., 2004; Yoshida et al., 2005, 2006).

For hormone perception in *Arabidopsis*, the E3 ubiquitin ligase that has overhauled the typical hormone receptor model is the SCF$^{TIR1}$ during auxin sensing. For the first time an E3 ubiquitin ligase was identified as a receptor that, upon signal binding, enhances targeting of its substrate for degradation. More specifically, an auxin-bound Transport Inhibitor Response (TIR) 1 F-box assembled into its E3 SCF conformation directs interaction with and ubiquitination of Auxin (AUX) or Indole-3-Acetic Acid (IAA) proteins for their destruction (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Without such heterodimerizing repressor proteins, auxin response transcription factors (ARFs)
would be free to activate auxin responsive genes and subsequent plant growth (Liscum and Reed, 2002; Dharmasiri et al., 2005b).

With the elucidation of this novel paradigm of small ligand perception, similarities between auxin perception and signalling and that of other hormones have become of interest (Tan and Zheng, 2009). In particular, attention has been paid to a SCF multiunit E3 ubiquitin ligase with a TIR1-distantly-related Coronatine Insensitive (COI) 1 F-box that may target Jasmonate-Zim-domain (JAZ) repressor proteins for degradation as directly promoted by JA binding during jasmonate signalling (Xu et al., 2002; Thines et al., 2007; Chini et al., 2007; Katsir et al., 2008) and a SCF multiunit E3 ubiquitin ligase with a Slepy (SLY) 1 F-box appears to target DELLA repressor proteins for removal as indirectly promoted by GA binding to DELLA-recruiting Gibberellin Insensitive Dwarf (GID) 1 receptor during gibberellin signalling (Dill et al., 2004; Ueguchi-Tanaka et al., 2005; Murase et al., 2008).

### 1.6.2 ABA and E3 ubiquitin ligases

The phytohormone abscisic acid (ABA) participates in many developmental and physiological processes including seed dormancy, stress acclimation, stomatal closure, and abscission (Finkelstein et al., 2002). E3 ubiquitin ligases have also been shown to be involved during ABA signalling. By modulating ABA-responsive transcription regulators ABA-Insensitive (ABI) 3 and ABI5 respectively, the RING E3 ubiquitin ligases ABI3-Interacting Protein (AIP) 2 and Keep on Going (KEG) have key roles during ABA responses (Zhang et al., 2005; Stone et al., 2006). In an interesting juxtaposition, while ABA yields accelerated ABI3 degradation, presumably through increased AIP2 transcript and protein accumulation (Zhang et al., 2005), ABA protects ABI5 from degradation, presumably through KEG (Lopez-Molina et al., 2002).

### 1.6.3 ABA: germination and abiotic stresses

The best known developmental ABA response is the role this phytohormone plays during seed germination inhibition. In conjunction with embryo maturation and vivipary prevention, seeds accumulate endogenous ABA to induce seed dormancy and this quiescent period needs to be broken before germination can occur (Finkelstein et al., 2002). Exogenous ABA is also capable of seed dormancy induction. By acting to
prevent precocious germination of seeds, ABA has been established as a hormone relevant during dormancy, and seed germination assays are convenient indicators of ABA signalling disruptions in mutant plants. Besides seed germination, ABA is also well known for its regulatory function during responses to environmental stresses. Increased ABA biosynthesis and accumulation have been correlated with salinity, drought, and cold signalling, and as such, promote abiotic stress tolerance (Finkelstein et al., 2002; Verslues and Zhu, 2005). Simultaneously, while activated ABA signalling is highly relevant during the transduction of different stress responses, ABA-independent response genes are also induced (Zhu, 2002), illustrating how complex the regulatory systems are when gene responses to environmental stresses are triggered.

To illustrate how the interplay between ABA and abiotic stress signalling can influence upon E3 ubiquitin ligase discovery and functional understanding, RING E3 Salt- and Drought-Induced Ring Finger (SDIR) 1 has been identified as a positive regulator of ABA signalling through which salt sensitivity and drought tolerance can be enhanced (Zhang et al., 2007). Likewise, DRE-Binding (DREB) 2 transcription factors downstream of ABI3 and ABI5 transcription regulators in the ABA signalling pathway that promote drought stress-protective gene expression are targeted for degradation by DREB2A-Interacting Protein (DRIP) 1 and DRIP2 RING E3 ubiquitin ligases (Qin et al., 2008)

1.7 Abiotic stress signalling

As sessile organisms, plants need to strategically acclimate their physiology in the short- and long-term upon onset of any of the wide variety of stresses that plants can encounter from the environment. Complicating plant molecular and cellular responses to these environmental assaults is the need to balance immediate changes required by the plant to minimize the physical damage brought upon by the stress with the continuing homeostasis objective for the plant to maintain optimal growth and development (Zhu, 2002). A wide and complex array of signalling pathways, from stress signal perception to secondary molecule signalling and necessary stress-responsive physiological changes, exist and the regulation of expression of stress-regulated gene sets is often involved (Xiong et al., 2002). Because the production of
stress-tolerance proteins require so much expenditure of a plant’s resources, precise specific induction of appropriate biological responses would be beneficial in the long-run while cross-talk between signal transduction pathways of some abiotic stress cues would yield immediate protective benefits (Knight and Knight, 2001).

1.7.1 Correlations between salt, drought, cold stress

Topically, salinity, drought, and low temperatures as stress signals are seemingly distinct, but amongst the multiple signalling cascades initiated, they share several related signal transduction branches (Xiong et al., 2002; Zhu, 2002). Because of the commonality of cellular dehydration induction among such osmotic-related stresses, it is not surprising that signalling from salt, drought, and cold stresses would have such common elements. Parts of such generalized stress signalling are an early transient increase in intracellular Ca\textsuperscript{2+} (Knight, 2000), the accumulation of osmoprotectants (Hasegawa et al., 2000), and the generation of regulatory molecules like ABA, ethylene, and JA (Xiong et al., 2002). While ABA involvement during abiotic stress has been clearly established (see above), the latter generation of ethylene and JA, hormones both traditionally defined for their involvement in senescence, abscission, and during fruit ripening for ethylene and during wounding for jasmonate, was the foundation to more recent discoveries of their contributions to abiotic stress responses (Hu et al., 2009; Lin et al., 2009). With the relevance of hormones during abiotic stress responses, comes the reiteration of the involvement E3 ubiquitin ligases have during the degradative regulation of the biosynthesis and signal transduction pathways associated with these hormones (see above). As an additional illustration of how intertwined the interactions of stress hormone signalling and of UPS regulation are beyond that of abiotic stress responses, the modulation of JA and ethylene signal transduction pathways has also been shown to be involved in defence gene expression in response to pathogen attack (Craig et al., 2009).

1.7.2 PUB proteins in abiotic stress responses

The first indication of PUB genes being up-regulated during abiotic stress responses came from a study in mangrove (Bruguiera gymnorrhiza) examining genes expressed in response to salt treatment (Banzai et al., 2002). The context of this study was to
identify salinity tolerance genes, given the ability of mangroves to survive in high salt growth environments. One PUB gene, BgBG55, was discovered to have a transient increase in expression in response to salt treatment. There are a large number of AtPUB genes that are up-regulated in the microarray expression data sets from salt treatment (Figure 2B, 2C), and this includes AtPUB6, AtPUB7, and AtPUB45, which are most closely related to BgBG55 (Samuel et al., 2006). Interestingly, there are other AtPUB genes that are more strongly up-regulated in the root with salt treatment including AtPUB19, AtPUB23, AtPUB24, AtPUB26, AtPUB27, AtPUB29, AtPUB48, and AtPUB58 (Figure 2C). The microarray expression data sets also show many of these AtPUB genes are up-regulated by other abiotic stresses such as cold, osmotic, drought, UV-B, wounding, and heat treatment (Figure 2B, 2C) as well. With AtPUB genes showing increased expression in response to a range of abiotic stresses (Figure 2B, 2C), the implication is that these proteins have a role in mediating ubiquitin-directed protein degradation in acclimation-related responses to various environmental stresses. The question then arises as to the nature of their biological functions during these abiotic stresses.

In hot pepper (Capsicum annuum L. cv Pukang), CaPUB1 was identified through its enhanced expression during dehydration, and transgenic Arabidopsis plants were used to assess the function of this gene. Over-expression of CaPUB1 in Arabidopsis showed increased drought and salt sensitivity (Cho et al., 2006). For the Arabidopsis CaPUB1 orthologs, AtPUB22 and AtPUB23, a similar correlation was seen with drought-sensitivity in over-expressing AtPUB22 and AtPUB23 plants versus drought-tolerance in the pub22 and pub23 double knock-out mutants (Cho et al., 2008). Based on the induction of these PUB genes in response to cold, drought, and salt treatments, it has been generalized that these E3 ubiquitin ligases may function as negative regulators during various abiotic stress responses (Cho et al., 2006, 2008).

AtCHIP is another PUB protein thought to have a role in environmental stress responses. AtCHIP is the sole TPR-repeat PUB, named after its sequence conservation to animal CHIP proteins, and, like the animal CHIPS, AtCHIP interacts with cytosolic HSP70 chaperones (Ballinger et al., 1999; Shen et al., 2007b). Because of its up-regulation upon temperature stress, AtCHIP is thought to coordinate cellular
responses to tolerate stress conditions (Dai et al., 2003; Yan et al., 2003; Qian et al., 2006). However, unlike CHIP over-expression which conferred stress protection and recovery in animal cells (Dai et al., 2003), Arabidopsis CHIP over-expression led to an increase in sensitivity to temperature stress, as well as the stress hormone ABA (Yan et al., 2003; Christmann et al., 2004; Luo et al., 2006). The first two AtCHIP-interactors to be identified were two regulatory A subunits, PP2AA3 and RCN1/PP2AA1, of the type 2A protein phosphatase (PP2A; Luo et al., 2006). PP2A is a ubiquitous protein phosphatase with links to broad functions in the plant, including carbon metabolism as well as ABA and stress responses (Kwak et al., 2002; DeLong, 2006). The rcn1 mutants have been previously found to have an ABA insensitivity phenotype as well as a decrease in PP2A activity (Deruere et al., 1999; Kwak et al., 2002). In contrast, plants over-expressing AtCHIP have increased PP2A activity alongside ABA sensitivity (Luo et al., 2006).

### 1.7.3 Other E3 ubiquitin ligases in abiotic stress responses

In Arabidopsis, evidence for the importance of the UPS during abiotic stress responses is starting to emerge. Besides PUB E3 ubiquitin ligases with connections to drought, salt, and temperature stress regulation, a RING E3 HOS1 appears to negatively mediate cold responses specifically through targeting of an ICE1 transcription factor (Lee et al., 2001; Dong et al., 2006). Correlating abiotic stress-related E3 ubiquitin ligases to their targeted substrates and/or their downstream stress-responsive gene transcription set is still very much in the early phase of our understanding of how plants cope with such stresses. Taking into account how complex and extensive abiotic stress signalling pathways are and how many environmental factors exist to trigger abiotic stress acclimation, it will become inevitable that, as researchers work to unravel the temporal regulation of abiotic stress-related genes, the relevance of UPS-based protein degradation becomes more pronounced.

### 1.8 Pathogen defences

Beyond having to adjust to the variety of abiotic stresses, plants also need to contend against the countless potential biotic assaults from the environment. Biotic challenges can come in many forms including those of bacteria, viruses, fungi, and insects (Dreher
and Callis, 2007). Should pathogens penetrate past preformed structural barriers like the cell wall and a layer of cuticular wax, plants quickly mount defensive responses when induced by invasion detection (Zeng et al., 2006). The inducible plant immunity generally falls into primary and secondary defence types, where the former is a frontline non-host resistance response and the latter provides a more pathogen-specific recognition in the event of primary defence breach through evasion and/or suppression. More specifically, the early pathogen perception occurs through the detection of structural components of microbes that have been conserved, and all the defence responses activated by the recognition of such Microbial- or Pathogen-Associated Molecular Patterns (MAMPs or PAMPs, respectively) through transmembrane pattern recognition receptors (PRRs) are together known as PAMP-Triggered Immunity (PTI) while the later pathogen perception occurs through the detection of effectors derived from pathogen invasion, and all the defence responses activated by the recognition of such infection-promoting avirulence (Avr) proteins through disease resistance (R) proteins inside the plant cell are together known as Effector-Triggered Immunity (ETI) (Craig et al., 2009). PTI is also known as basal or innate resistance while ETI is also known as the R-gene-mediated or gene-for-gene resistance, which has since been refined into the “guard hypothesis”, whereby R proteins indirectly recognize the pathogen Avr only after complexing with host proteins known as virulence/effector targets (Dangl and Jones, 2001).

1.8.1 Correlations between PAMP- and effector-triggered immunities
The plant defensive response to pathogens is generally characterized by host systemic acquired resistance (SAR) induction, defence-related gene expression, and/or preventative cell destruction, all of which are likely induced by various signalling phenomena (ex. SA, ROS, calcium) triggered by infection (Lam et al., 1999; Kuriyama and Fukuda, 2002; Hoeberichts and Woltering, 2003; van Doorn and Woltering, 2005). Upon recognition of PAMPs (or MAMPs), initiation of signalling cascades leading to defensive responses is swift and these include cytosolic calcium (Ca^{2+}) fluctuations, production of reactive oxygen species (ROS) known as the oxidative burst, and salicylic acid (SA) accumulation (Craig et al., 2009). Microarray experiments have allowed the analysis of the amplitude of transcriptional variations upon pathogen perception, and
many changes in gene expression have been found to overlap between PTI and ETI, and, in addition, the downstream transcriptome changes are often shared by induction from PAMPs from different pathogens (Zipfel et al., 2006). The speed with which plants trigger their primary basal immunity suggests that proteins involved in PTI signal transduction pathways pre-exist and readily await the arrival of pathogens to activate post-translational modifications of these proteins to modulate defence responses (Stulemeijer and Joosten, 2008). While primary defences initiated by PTI are notable for their rapid induction, generally, secondary ETI signal transduction is regarded as a more amplified version of PTI. The intensified ETI defence signalling is punctuated by *Pathogen-Related (PR)* genes transcription and brings on global changes like SAR and local alterations like the hypersensitive response (Zeng et al., 2006).

### 1.8.2 PUB proteins in pathogen defence responses

The plant function with the most PUB associations has been related to pathogen defence and the hypersensitive response (HR). The first example emerged from the *Avr9/Cf-9 Rapidly Elicited (ACRE)* gene expression analysis in tobacco (*Nicotiana tabacum*) where genes showing up-regulation in response to treatment with the fungal pathogen *Cladosporium fulvum* (Cf)-derived Avr9 peptide were identified. *NtACRE74* and *NtACRE276* were two PUB genes discovered in this study (Durrant et al., 2000). As well, U-box genes in parsley (*PcCMPG1*), and *Arabidopsis* (*AtPUB20/CMPG1*) were shown to be early defence response genes activated immediately after pathogen-derived elicitor treatment (Kirsch et al., 2001; Heise et al., 2002). Another study was subsequently conducted in *Arabidopsis* to identify *Flagellin Rapidly Elicited* (FLARE) genes which show increased expression in response to treatment with the flg22 peptide, a conserved component of bacterial flagellin involved in activating the innate immunity response (Navarro et al., 2004). *AtPUB20/AtPUB21 (CMPG1, NtACRE74)* and *AtPUB17 (NtACRE276)* were also present in the FLARE data set along with *AtPUB12* and *AtPUB5* (Navarro et al., 2004). In fact, many *AtPUB* genes are observed to be up-regulated in response to various pathogen and elicitor treatments (Figure 2D), and the involvement of these proteins in plant defence responses will likely prove to be an even more common phenomenon in this family.
Both CMPG1 and ACRE276/PUB17 were subsequently confirmed to be U-box E3 ubiquitin ligases which act as positive regulators of the HR in response to pathogenic infections (Gonzalez-Lamothe et al., 2006; Yang et al., 2006). When NtCMPG1 (NtACRE74) was silenced in Nicotiana benthamiana, there was a reduced HR following treatment with Avr9 while over-expression of NtCMPG1 resulted in a stronger HR following Avr9 treatment. As well, the silencing of SICMPG1 in tomato (Solanum lycopersicum) led to decreased resistance to C. fulvum (Gonzalez-Lamothe et al., 2006). Similarly, there was an association between silencing NtACRE276 with reduced HR and silencing SlACRE276 with decreased resistance to C. fulvum (Yang et al., 2006). Transgenic expression of the most similar Arabidopsis protein to NtACRE276, AtPUB17 (previously identified by BnARC1 similarity), in ACRE276-silenced tobacco plants rescued the HR. As well, pub17 knock-out Arabidopsis plants displayed decreased resistance to avirulent Pseudomonas syringae pv. tomato (Pst) (Yang et al., 2006).

The identification of CMPG1 and ACRE276/PUB17 proteins as positive regulators of the HR is in juxtaposition with the regulatory role uncovered for the rice (Oryza sativa) Spotted Leaf 11 (SPL11) gene. SPL11 was found to be negative regulator of HR-associated leaf lesion formation and defence against pathogens (Zeng et al., 2004). The spl11 mutation was originally identified as a rice lesion mimic mutant (Singh et al., 1995), which is characterized by the spontaneous appearance of disease lesions formed in the absence of pathogenic stimuli (Lorrain et al., 2003). Further characterization revealed increased resistance to various fungal and bacterial pathogens (Yin et al., 2000), and the SPL11 protein identity as a PUB and activity as an E3 ubiquitin ligase was subsequently determined (Zeng et al., 2004). For the OsSPL11-related Arabidopsis genes AtPUB12, AtPUB13, and AtPUB14, the phenotypic analysis of knock-out lines uncovered a stunted growth phenotype and non-pathogen induced cell death (Zeng, 2005).

Another group of negative regulators of plant defence responses, AtPUB22, AtPUB23, and AtPUB24, was recently discovered by Trujillo et al. (2008). These genes show increased expression following flg22 treatment as well as infection with bacterial pathogen Pseudomonas syringae and fungal pathogen Hyaloperonospora parasitica
The Arabidopsis pub22 pub23 double mutant showed increased oxidative burst, a plant defence response involving a rapid and temporary generation of ROS (Trujillo et al., 2008). The inclusion of a third knock-out, pub24, further enhanced this response, and the pub22 pub23 pub24 triple knock-out showed increased resistance to bacterial and oomycete pathogens with increased oxidative bursts and HR cell death occurring as part of this response (Trujillo et al., 2008). Interestingly, the pub22 pub23 double knock-outs were previously shown to have increased drought tolerance (Cho et al., 2008; described below), and thus, it appears that these PUB proteins act as negative regulators of both abiotic stress and plant defence responses.

Most recently, two functionally redundant PUB E3 ubiquitin ligases were identified for their positive regulation of both innate and R-gene-mediated resistance (Monaghan et al., 2009). MOS4-Associated Complex (MAC) 3A/AtPUB59 and MAC3B/AtPUB60 were identified from immuno-purifications facilitated by a component of the MAC nuclear protein complex characterized through Modifier of snc1 (MOS) genes as positive regulators of plant immunity (Monaghan et al., 2009). MOS genes themselves were identified through a suppressor screen to search for signalling components downstream of Suppressor of npr1-1, constitutive (SNC) 1, the latter of which, as its name surmises, was identified as an R protein through its suppression of mutations in Non-expressor of PR (NPR) 1 protein which display repressed pathogen susceptibility (Li et al., 2001).

The Arabidopsis mac3a/pub59 mac3b/pub60 double mutant showed defects in primary and secondary pathogen defence pathways, ranging from increased bacterial growth after infection to impaired enhanced resistance (Monaghan et al., 2009).

### 1.8.3 Other E3 ubiquitin ligases in pathogen defence responses

Amongst the responses altered by jasmonate-mediated signalling through the activated SCF\(^{C01f}\) multiplex E3 ubiquitin ligase are plant defences against insects and microbial pathogens (McConn et al., 1997; Thomma et al., 1998; Turner et al., 2002; Devoto et al., 2005). Similarly, amongst the genes transcriptionally controlled by ethylene-mediated signalling through the deactivation of SCF\(^{EBF1/2}\) multiplex E3 ubiquitin ligases are defence-related and PR proteins (Dreher and Callis, 2007). Without the presence of ethylene or at over-saturated ethylene levels, the EIN3 Binding F-box (EBF) 1 and
EBF2 F-boxes target the constitutively expressed Ethylene Insensitive (EIN) 3 positive transcription factors for degradation (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004; Binder et al., 2007). Other E3 defence regulators include Suppressor of nim1/npr1 (SON) 1, a negatively regulating F-box protein identified through its presumed targeting of constitutive activators of SAR-independent defence responses (Kim and Delaney, 2002).

1.8.4 E3 ubiquitin ligases as pathogen virulence factors

With the prominent numbers of E3 ubiquitin ligases that have been identified in plants and their potential for thousands of targets, one notable speculation behind why E3 ubiquitin ligases have undergone such a selective expansion in plants is the possible use of this amplified UPS component as a form of rapidly evolving plant innate immunity to recognize the variety of proteins found within invading pathogens (Vierstra, 2009). This is not to say that co-evolving plant pathogens have been passively idle while plants mount their counterattacks. Some bacterial pathogens have come to exploit a host plant’s existing ubiquitin proteasome system for their own benefit. The plant pathogen, Pseudomonas syringae pv. tomato DC3000, uses a type III secretion system to inject the AvrPtoB effector into host cells where this bacterial virulence factor mediates the suppression of the plant immune response by evading the HR and basal defence responses (Abramovitch and Martin, 2005; de Torres et al., 2006). This bacterial effector was found to be a U-box E3 ubiquitin ligase that is functional in plant hosts where the ligase activity of a structurally similar U-box was necessary to inhibit cell death and immunity in plants and ensure bacterial virulence (Janjusevic et al., 2006; Abramovitch et al., 2006). Thus, as a U-box E3 ubiquitin ligase that is translocated into plant host cells, bacterial effector AvrPtoB is an example of a pathogen evolving molecular mimicry of host proteins, and may provide further insight into the functions of PUB proteins.

Tomato Fen is the proposed substrate for AvrPtoB, the bacterial E3 ubiquitin ligase used by the Pseudomonas syringae pathogen to hijack the plants ubiquitination system to turn against the host in favour of the invader. Following the scenario that bacterial U-box protein AvrPtoB could be targeting plant proteins necessary for activating plant
defence responses, a dominant-negative form of AvrPtoB was used to pull out Fen and shown to be the target of this effector protein (Rosebrock et al., 2007). Fen is related to the Pto kinase resistance gene family (Martin et al., 1994). The original Pto kinase was found on its basis to confer plant immunity against pathogenic infection by recognizing specific avirulence proteins and mounting an assortment of plant defence responses that was dependent on kinase activity (Pedley and Martin, 2003). But while Fen has been shown to be an active kinase (Chang et al., 2002), research on Fen’s role during pathogenic resistance has been limited. The discovery that plant protein Fen is a bacterial E3 ubiquitin ligase target substrate during plant defence evasion and the subsequent immunity activation characterization have confirmed the involvement of Fen as a positive regulator during plant immune responses in tomato (Rosebrock et al., 2007). Thus, it appears bacterial E3 effector AvrPtoB targets Fen for ubiquitin-mediated degradation and as a result inactivates Fen and the plant defences normally elicited by this kinase, allowing for pathogen invasion.

1.9 PUB proteins during programmed cell death – an underlying theme?

One of the themes emerging from E3 ubiquitin ligase research is the regulation of cell death as a frequent element of ubiquitin ligase action. Programmed cell death differentiates itself from necrosis in that, unlike the latter, where cell death is often passive and the cause of death is primarily damage caused by injury to the cell (ex. mechanical), cell death in the former is actively regulated and results from developmental cues or induction stimuli. In plants, programmed cell death is an integral part of plant growth and development including seed and embryo development; leaf (shape, senescence), stem (tracheary elements) and root (root cap, aerenchyma) development; and flowering and reproduction (anther dehiscence, female gametophyte development, self-incompatibility, and petal senescence) (Rogers, 2005). As well, cell death occurs as part of plant responses to biotic and abiotic stresses (Love et al., 2008; Reape et al., 2008). As such, it would not be farfetched to assume that, given their large numbers and variety, PUB proteins might be involved in many of these processes.
1.9.1 Hypersensitive response – a pathogen response involving PCD

In plants, the best-studied occurrence of programmed cell death (PCD) is the HR, a form of triggered immunity exhibited during certain plant-pathogen interactions. In resistant plants, a rapid resistance response is mounted in defence against avirulent pathogens and HR is associated with this response (van Doorn and Woltering, 2005; Greenberg and Yao, 2004; Heath, 1998). The cell death associated with HR is visualized by dry lesions, formed at and surrounding the site of attack, which are dead cells that are clearly defined from surrounding healthy cells (Lam et al., 2001; Pennell and Lamb, 1997). Thus selective host cell suicide, accompanying the plant resistance response, appears to eliminate the spread of infection by helping to contain pathogen attack. In comparison to the plant succumbing to disease, the sacrifice of a few cells for the health of the whole plant makes HR desirable for pathogen resistance. PUB E3 ubiquitin ligases with altered HR or HR-like phenotypes include the spl11 mutation conferring a spontaneous cell death lesion mimic in rice (Yin et al., 2000; Zeng et al., 2004); the NtCMPG1/NtACRE74-silenced or NtACRE276-silenced tobacco plants and SICMPG1-silenced or SIACRE276-silenced tomato plants showing reduced HR upon elicitor treatment and stronger HR in tobacco plants over-expressing NtCMPG1/NtACRE74 (Gonzalez-Lamothe et al., 2006; Yang et al., 2006); spontaneous leaf chlorosis in atpub12, atpub13, and atpub14 knock-outs (Zeng, 2005); increased chlorosis in atpub17 knock-outs upon avirulent Pst inoculation (Yang et al., 2006); and enhanced cell death in leaves of pub22 pub23 pub24 triple mutant seedlings infiltrated with Pst (Trujillo et al., 2008).

1.9.2 Leaf senescence – a light response involving PCD

One of the more controversial instances of PCD is leaf senescence, a process exhibited during the final stage of leaf development associated with demise of the leaf. The cell death associated with leaf senescence is visualized by yellowing leaves caused by the degradation of green-pigmented chlorophyll, leaving behind the yellow-red-pigmented carotenoids. It is the rare reversibility of leaf yellowing prior to a point of no return (Zavaleta-Mancera et al., 1999a; Zavaleta-Mancera et al., 1999b) and the organ-level event of leaf senescence (vs PCD's cell-individualized focus) that contribute to the debate surrounding whether senescence is a term suited to be fully synonymous with,
similar to, or distinctively unrelated from PCD (Thomas et al., 2003; van Doorn and Woltering, 2004). Semantics aside, what is agreed is that leaf senescence is a regulated program that, like all other PCD phenomenon, involves a series of active events characterized by changes in cellular gene expression, controlled cellular degeneration, and reallocation/recycling of resources to tissues not undergoing cell death that all eventually lead to the death of the leaf (Guo and Gan, 2005).

Leaf senescence can be brought about by many environmental factors, including water deprivation, unfavourable temperatures, and nutrition deficiency, but most notably when shading or darkness make lighting conditions insufficient for these photosynthesis-specialized organs (Guo and Gan, 2005). It makes senses that a consistently shaded leaf would reallocate its nutritional resources to another more light-energy accessible leaf to optimize overall photosynthetic efficiency of the whole plant. Photomorphogenesis, circadian clock function, and photoperiodic flowering are all light-related signalling pathways that appear to be regulated in part by E3 ubiquitin ligase-mediated proteolysis in Arabidopsis (Henriques et al., 2009) and most recently a PUB protein has been identified as a negative regulator of premature leaf senescence under low light conditions (Raab et al., 2009). Grown under high photon flux density (PFD), leaves of Arabidopsis plants with null mutations in the Senescence-Associated E3 Ubiquitin Ligase 1 (SAUL1/PUB44) gene stayed green, while leaves of these same saul1/pub44 mutants displayed yellowing in low PFD, as accompanied by decreased chlorophyll content and senescence-associated gene (SAG) up-regulation, all hallmarks of leaf senescence (Raab et al., 2009).

This involvement of a PUB in mediating certain light signal transduction pathways in response to light-level adjustments is not new. Besides being responsive to light levels that are too low, leaves are also sensitive to light levels that are too high, whereby both of such opposite and extreme intensities would decrease the photochemical efficiency of photosystem II (PSII). Recent research on other target proteins of AtCHIP has revealed an additional and novel role for AtCHIP in the regulation of protein degradation in the chloroplast where localized leaf variegation was seen in high-light treated Arabidopsis plants over-expressing AtCHIP (Shen et al., 2007b);
AtCHIP was found to interact with two protease subunits, ClpP4 and FtsH1 (Luo et al., 2006; Shen et al., 2007a, b). ClpP4 is a core subunit of the Clp protease which is involved in degrading proteins in the chloroplast stroma (Shen et al., 2007a). FtsH1 is a subunit of the FtsH protease complex which is believed to degrade mis-folded or damaged membrane proteins in the chloroplast thylakoid membranes as part of the repair mechanism during photoinhibition damage (Shen et al., 2007b). Interestingly, the AtCHIP-over-expressing plants displayed both a cell death phenotype and increased production of ROS in leaves when grown under the high-intensity light conditions (Shen et al., 2007b). In the case of FtsH1, the conditional cell death of AtCHIP-over-expressing plants is thought to be due to the reduced FtsH protease activity which is correlated with increased levels of one of the FtsH protease substrates, damaged D1 protein, and in turn is thought to cause over-production of ROS (Shen et al., 2007b). As the PSII reaction centre protein, D1 needs to be replaced upon being damaged by photoinhibitory illuminaton, and their lack of removal would result in reversible inhibition of PSII activity. From these results, the idea is presented that AtCHIP targets the FtsH protease complex under normal conditions, but not under high-light stress where the protease complex is involved in repair of PSII oxidative photorecovery (Sakamoto et al., 2003). In the case of ClpP4, AtCHIP is suggested to promote ClpP4 degradation under high-intensity light stress conditions, and as a result, down-regulate the Clp protease under these conditions (Shen et al., 2007a). The Clp protease in turn has been found to degrade chlorophyllide a oxygenase (CAO), an enzyme responsible for chlorophyll b biosynthesis (Nakagawara et al., 2007). The synthesis of chlorophyll b by CAO is dynamically regulated to optimize the absorption of light energy by photosystem II under differing light conditions (Tanaka et al., 2001). While the Clp protease acts as a negative regulator of CAO under differing light conditions and chlorophyll b levels, it does remains to be seen if AtCHIP plays a part in the dynamic control of photosynthetic complexes to gather sufficient light energy.

1.9.3 ABA — a potential regulator of abiotic, biotic, and senescence-directed PCD

The role of ABA as the plant abiotic stress hormone and as the mediator of plant acclimations to abiotic stress has been well acknowledged. Increased biosynthesis and
accumulation of ABA resulting from detection of abiotic stresses has been correlated to downstream stress tolerance responses, including stomatal aperture modifications and stress-responsive gene expression (Verslues and Zhu, 2005). Simultaneously, a growing amount of research is documenting the complex role ABA has as a plant biotic stress hormone. Not as straightforward or well understood as the promotion of abiotic stress acclimations, ABA appears to contribute both synergistically and antagonistically to plant defence during disease resistance, with its role dependent on pathogen-type and host-entry strategy of location and timing, initially being stimulatory pre-invasion then becoming suppressive post-invasion and during late disease resistance (Ton et al., 2009).

An explanation behind the purpose of such elaborate and apparently contradictory functions of ABA during defence responses has yet to clearly emerge. One possible explanation is that ABA-mediated promotion occurs during early pathogen invasion and not during late penetration resistance when costly JA- and SA-dependent defences would be activated (Ton et al., 2009). Such a cost-benefit strategy could also be applied to the antagonistic interplay between abiotic and biotic stress signalling as exemplified by the need to balance plant stress-responses towards relevant signalling networks while simultaneously down-regulating other networks with less momentary precedence to avoid unnecessarily generating proteins involved for both abiotic and biotic defence responses. Although abiotic and biotic stresses originate from the same external environment of the plant, they are different stresses requiring variations in stress signalling to specify the appropriate response to each type of stress, and by regulating both abiotic and biotic responses, ABA may be the hormone to shift the plant-stress response priority towards abiotic stress acclimation in its elevated presence and, in its deficiency, towards biotic defence responses (Asselbergh et al., 2008).

While abiotic and biotic stress responses are integrated and coordinated differently from each other, overlap between the responses to abiotic and biotic stresses does exist and remains to be fully characterized. Some shared similarities can be justified by the common use of intersecting signalling components, most notably at the signal perception level, when biotic stress caused by the colonization of pathogens results in abiotic-stress-like physiological and cellular changes (Asselbergh et al., 2008). Leaf
senescence onset and progression, with emerging evidence showing ABA signalling being involved to enhance such events (Raab et al., 2009), can be induced by both abiotic and biotic stresses (Guo and Gan, 2005). As such links between senescence induction, ABA, and stress are being predicted (Yang et al., 2003; Munne-Bosch and Lalueza, 2007).

The revelation that oxidative stress-induced senescence more closely resembles the HR than developmental senescence (Wingler and Roitsch, 2008) suggests that an underlying point of convergence between abiotic and biotic stress, and senescence induction with ABA signalling may be the production of ROS (Fujita et al., 2006). ROS involvement occurs through oxidative burst of the plant defence responses during biotic stress, through oxidative stress-related generation of ROS during certain abiotic stresses like light, cold, and drought, through ROS formation during ABA-induced stomatal closure, and through ROS accumulation as an indicator of leaf age and inducers of SAGs during senescence regulation (Apel and Hirt, 2004, Laloi et al., 2004). The integration of ABA into the ROS signalling pathway to govern abiotic and biotic stress responses that yield PCD-related HR and leaf senescence has yet to be fully resolved (Christmann et al., 2006), but it would be of interest to see whether, like the ABA-induced resistance promotion or susceptibility increase during stress-signalling, the similarly contradictory PUB-regulation during some plant-pathogen interactions might also function as conversion points allowing priority shifting directing proteolysis to select responses during biotic or abiotic stress acclimation.

1.10 PUB proteins & the 26S proteasome

The 26S proteasome is generally known as the final destination of the proteins tagged for destruction by the ubiquitin moieties conferred upon specific target substrates by E3 ubiquitin ligases like PUBs during the ubiquitination process. The large 26S proteasome itself, however, is also made up of a complex of proteins which, in plants, appear to have no exception from the directed-ubiquitination-type of protein regulation, ironically, designed to bring targeted proteins to it, specifically through the U-box E3 ubiquitin ligases. Some perplexing aspects are why PUB22, PUB23, and PUB24 are targeting subunits of the 19S regulatory particle, and why the loss of these PUB proteins
is correlated with increased tolerance or resistance to various stresses while increased expression of these genes is observed in response to these same stresses (Figure 2; Cho et al., 2008). The consequence of reducing the levels of the 19S regulatory particle can be seen in *Arabidopsis* loss-of-function mutants for the RPT2a, RPN10, and RPN12a subunits (Kurepa et al., 2008). These mutants were found to have reduced levels of the 26S proteasome, and this was associated with increased sensitivity to high temperature stress attributable to a reduction in the ubiquitin-mediated degradation of mis-folded proteins (Kurepa et al., 2008). Thus, the observed dissociation of the RPN12a subunits from the 19S regulatory particle seen in plants over-expressing either *AtPUB22* or *AtPUB23* would be consistent with a reduction in 26S proteasome function which is needed for environmental stress responses (Cho et al., 2008).

Another phenotype observed in the *rpt2a*, *rpn10*, and *rpn12a* *Arabidopsis* mutants was increased oxidative stress protection that could be correlated to 19S regulatory particle assembly being affected (Kurepa et al., 2008). This is attributed to the fact that proteolysis by the 20S core particle is associated with ubiquitin-independent degradation of oxidized proteins and can occur without the regulation of the 19S particle. This oxidative stress tolerance phenotype of such knock-down mutations of putative PUB22/23 substrates is consistent with the observed increase in ROS generation in the *pub22 pub23 pub24* triple knock-out mutants (Trujillo et al., 2008) and an intact 19S coupled with the 20SP to make 26S proteasomal units could be the basis of the increased drought resistance seen in *pub22 pub23* double knock-outs (Cho et al., 2008). Additionally, oxidized proteins as a result of ROS present in the cellular environment act as signals to activate cell death seen during plant immunity (Trujillo et al., 2008). In compensation for increased drought tolerance, it could be predicted that the latter mutants would exhibit oxidative stress hypersensitivity. What has been suggested is that by having E3 ubiquitin ligases modulate the ubiquitin-proteasome pathway itself, through the 19S regulatory particle subunit-targeting action of certain PUBs to alter the regulatory aspect of the 26S proteasome, this self-regulatory loop is a way to finely adjust the ubiquitin-dependent and ubiquitin-independent protein degradation governing any cellular functions that need to be balanced in response to plant acclimation to any general stresses.
1.11 Bioinformatic approaches

There is a strong probability that the UND-containing AtPUB-ARMs may operate in a partially redundant manner, as shown for other PUB proteins (Cho et al., 2008; Trujillo et al., 2008), making functional characterization of this family more onerous. The computational approaches used in bioinformatics have helped functional genomics by allowing the user to narrow the scope of the experiments used in determining gene function. Various user-friendly web-based Arabidopsis functional genomics programs have made the data generated from microarray projects more easily accessible, most notably the Bio-Array Resources (BAR) for Plant Functional Genomics located at http://bar.utoronto.ca (Toufighi et al., 2005) for its use in this thesis and others such as Genevestigator located at www.genevestigator.com (Hruz et al., 2008).

1.12 Thesis Objectives

Ultimately, the main objective of this thesis was to identify biological functions for two Arabidopsis PUB-ARM family members, AtPUB18 and AtPUB19, during plant growth, development, and/or acclimation. Because of their increased gene expression during certain abiotic stresses, it is hypothesized that AtPUB18 and AtPUB19 are E3 ubiquitin ligases whose directed-degradatory function becomes important during the regulation of the signal transduction pathways activated in times of abiotic stress induction. AtPUB18 and AtPUB19 are two closely related members of the AtPUB-ARM family and the elucidation of any potential plant functions attributed to these proteins was investigated as follows: 1) the direct morphological characterization of T-DNA insertion mutants, 2) the characterization of AtPUB18 and AtPUB19 genes through expression studies, and 3) conditional assays, used to draw out any increased or decreased sensitivity of the T-DNA knock-out lines to abiotic stresses.

Observations of T-DNA insertion lines in AtPUB18 and AtPUB19 did not show any altered developmental phenotypes while knock-out plants were grown under normal growth conditions, corroborating the strong probability that AtPUB18 and AtPUB19 may operate in an at least partially redundant manner. To alleviate any possible functional redundancy, double pub18 pub19 mutants were generated. In the course of generating initial double crosses between AtPUB18 and AtPUB19 knock-outs, the appearance of
collapsed non-viable pollen in anthers and aborted ovules in siliques was seen from plants harbouring one double heterozygous combination. In addition, genotype screening of the progeny from this selfed double heterozygous combination yielded numbers demonstrative of non-independent gene assortment. A subsequent double mutant from an alternate double cross did not substantiate these phenotypes and an attempt to conditionally complement the phenotypes in the original double heterozygote proved unsuccessful, insinuating that these phenotypes were attributable to an alternate T-DNA insertion. Upon the generation of double homozygous plants, various growth, germination assays, and root length assays were done to assess any differences in plant development and stress responses with the loss of AtPUB18 and AtPUB19.
CHAPTER 2: MATERIALS AND METHODS

2 Materials and Methods

2.1 Plant growth conditions

*Arabidopsis thaliana* wild-type (Col-0) and transgenic plants with Col-0 background (see below) were grown on professional general purpose growing medium potting soil (Premier Pro-Mix®) supplemented with water soluble fertilizer with micronutrients (6mL per 5L of water; Plant Prod® 20-20-20 All Purpose Fertilizer) at 22°C under long-day conditions (16hr light / 8 hr dark photoperiod) in a growth room. Seeds were surface-sterilized by immersion in 70% ethanol for 2 minutes, followed by 8 minutes in a sterilization solution (30% (v/v) bleach, 0.02% (v/v) Triton X-100 (Sigma)). To remove all traces of the sterilization solution, the seeds were then rinsed 5-10 times with sterile distilled deionized water (ddH2O). For growth on agar plates, seeds were sown onto media (0.5x Murashige and Skoog (MS) salts (Caisson Laboratories), 0-0.05% (w/v) MES (4-morpholine-ethanesulfonic acid, ICN Biomedicals), 0-1% (w/v) sucrose, 0.4-1% (w/v) phytoagar (Phytoblend, Caisson Laboratories), pH 5.7-5.8 (adjusted with KOH)), stratified at 4°C in the dark for at least 3 days and grown in a growth chamber under long-day conditions at 22°C (Percival Scientific) before seedlings (with fully emerged true leaves) were transferred to soil. Alternatively, seeds were stratified in microtubes and directly sown onto soil. In the Percival growth chamber, the fluence rate of white light was 90-100μE s⁻¹ m⁻² as measured using LI-185B light meter combined with a quantum sensor (LI-COR). For the high-light root growth assays, the same temperature and photoperiod conditions were used in additional growth chamber used (Percival Scientific) but light levels were set to 300-350μE s⁻¹ m⁻².

2.2 Bioinformatics

For sequence comparisons, Basic Local Alignment Search Tool (BLAST) 2 was used at TAIR (www.arabidopsis.org/Blast). For multiple sequence alignment, ClustalW run locally on BAR (www.bar.utoronto.ca) was used (http://bar.utoronto.ca/ntools/cgi-bin/ntools_multiplealign_w_mview.cgi). To create absolute and relative “electronic fluorescent pictographic representations”, *Arabidopsis* eFP Browser from BAR
For Arabidopsis chromosomal map generation, the Chromosome Map Tool on TAIR was used (www.arabidopsis.org/jsp/ChromosomeMap/tool). For generation of expression level heat maps, the DataMetaFormatter genomic tool found in BAR was utilized (http://bar.utoronto.ca/ntools/cgi-bin/ntools_treeview_word.cgi). To obtain the expression level data used later in the DataMetaFormatter in raw text format, the e-Northerns with Expression Browser tool from BAR (http://bar.utoronto.ca/affydb/cgi-bin/affy_db_exprss_browser_in.cgi) was used to generate “Average of replicate treatments relative to average of appropriate control” ratio results; from the same Expression Browser output, the graphical representation of log transformed clustered data showing the average linkage hierarchical clustering and the Pearson correlation coefficient was also used (Toufighi et al., 2005). After selective editing, the raw text data were resubmitted into the DataMetaFormatter genomic tool (http://bar.utoronto.ca/ntools/cgi-bin/ntools_treeview_word.cgi) to generate new e-Northern heat maps.

For comparative sequence analysis, distance-based phylogenetic analyses were performed with the neighbour-joining algorithm implemented in MEGA3 (Kumar et al., 2004) on the UND-containing AtPUB-ARMs. Trees built on the basis of the similarity between the ARMs protein sequences were generated by rooting at the mid-point. The phylogenies were reconstructed using the Poisson correction substitution model with 1000 bootstrap replicates. Sequences were initially aligned by the ClustalW standard method (Higgins et al., 1996). The multiple sequence alignment parameters included the Gonnet protein weight matrix with residue-specific and hydrophilic penalties. For the ARM-based alignment, the region C-terminal to the U-box was considered. The positions for the U-box domain in each sequence were obtained from the profiles stored in the Pfam collection (http://pfam.sanger.ac.uk; Bateman et al., 2002) as accessed through MatDB (Schoof et al., 2002). To optimize sequence similarity, manual adjustments to the alignments were also performed.
2.3 *Arabidopsis* T-DNA insertion lines

The AGI gene identifiers of *AtPUB18* and *AtPUB19* are At1g10560 and At1g60190, respectively. Each gene (from the start to stop codon) comprises 2094bp and 2061bp, to generate proteins with 697 and 686 amino acids, respectively. Homozygous *pub18* and *pub19* mutants were isolated by selfing the plants grown from the seeds originally obtained from T-DNA insertion lines found in the Syngenta *Arabidopsis* Insertion Library (SAIL) resource, the Salk Institute Genomic Analysis Laboratory (SIGnAL) project ([http://signal.salk.edu](http://signal.salk.edu)) (both as available from the *Arabidopsis* Biological Resource Center (ABRC)), and the GABI-Kat (Genomanalyse im biologischen system pflanze (Genome analysis in the plant biological system) - Kölner *Arabidopsis* T-DNA) project ([http://www.mpiz-koeln.mpg.de/GABI-Kat](http://www.mpiz-koeln.mpg.de/GABI-Kat)) collections. Available seed stocks were identified by Polymorphism/Allele search at The *Arabidopsis* Information Resource (TAIR- [www.arabidopsis.org](http://www.arabidopsis.org)). Stock numbers SAIL_634_G01, SALK_001831, and GABI-Kat_428E05 correspond to *pub18-1*, *pub18-2*, and *pub18-3* insertion mutations in *AtPUB18* respectively. Stock numbers SALK_152677, SALK_058791, and GABI-Kat_099D10 correspond to *pub19-1*, *pub19-2*, and *pub19-3* insertion mutations in *AtPUB19* respectively. Information on insertion sites (location, direction) taken from the stock sites were noted and then confirmed or reassigned upon sequencing across the insertion site of each T-DNA line.

2.4 Plant genomic DNA extraction

Genomic DNA (gDNA) isolation was performed using fresh or liquid-nitrogen frozen *Arabidopsis* tissue. Generally, a leaf (3-4mm diameter) or singular/pooled seedlings were used. Tissue was homogenized in 250μl of CTAB buffer (0.55M cetyl-trimethyl-ammonium bromide (CTAB), 1M sodium chloride (NaCl), 0.02M ethylenediaminetetraacetic acid (EDTA) pH8.0, 0.1M (hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH8.0, with freshly added 0.4% β-mercaptoethanol) and incubated at 60-65°C for 30-60 minutes. An equal volume of chloroform was added to each sample, gently vortexed and inverted to mix, before being centrifuged for 5 minutes at 13,000 rpm at room temperature. The aqueous phase was transferred to a sterile tube and 200μl of isopropanol was added. Samples were inverted to mix and left to sit at room temperature for 15 minutes. After incubation, samples were centrifuged at
room temperature at 13,000 rpm for 20 minutes. Supernatant was discarded and the pellet washed with 70% ethanol and air dried at room temperature overnight. DNA pellets were resuspended in 40 µl ddH2O. Samples were stored at -20°C.

2.5 Genotypic screening using PCR

Homozygous mutants were identified through polymerase chain reaction (PCR) genotypic screening using primers specifically designed to amplify across the T-DNA insertion site. The PUB18-specific primers used to genotype for the pub18-1 mutant were 5’-TCAGAGAAAACTCTCAGGCGGA-3’ and 5’-CCAGACAAGACCTATTAAAAACTG-3’; for the pub18-2 mutant, 5’-CATCGTTTTGCGGGGATTAGT-3’ and 5’-GTGAACGAACCGTCCTCTTGA-3’; for the pub18-3 mutant, 5’-TGGTTGATAACGTATCGAG-3’ and 5’-CTGGAACCTCAGGATCATTCTTG-3’. The PUB19-specific primers used to genotype for the pub19-1 mutant were 5’-GAGAATGATCAAGGGAGACGA-3’ and 5’-ATTACAATAGTATAGCTCTTTGGG-3’; for the pub19-2 mutant, 5’-CTTTTTACAGATCGTGTTTAGTGG-3’ and 5’-ATTACAATAGTATAGCTCTTTGGG-3’; for the pub19-3 mutant, 5’-GGTATACTTCAGGACGATTTGTTTACAG-3’ and 5’-CCCCACGACGGGACTGAT-3’. For the T-DNA left border primer, 5’-AATTTCATAACCAATCTCGATACAC-3’ was used for the SAIL line (pub18-1), 5’-TTTTTCGCCCTTTGACGTTGGA-3’ was used for SALK lines (pub18-2, pub19-1, pub19-2), and 5’-CATTTGGGAAGGATGTAAC-3’ was used for the GABI-Kat lines (pub18-3, pub19-3). To determine the genotype of plants, two primer sets were utilized: the first was a gene-specific set consisting of the forward and reverse gene-specific primers, the second being a T-DNA specific set consisting of one of the gene-specific primers paired with the left-border of the T-DNA insert.

Each PCR reaction consisted of the following: 1x Tsg buffer, 1.6mM MgSO4, 0.2mM dNTPs, 0.48µM of each primer, 0.8% dimethyl sulfoxide (DMSO), and 1 unit Tsg polymerase (BioBasics), 1µg of gDNA. The PCR Thermo cycler (PCR Express PCYL220, Thermo Hybaid) program was set as 94°C for 3 min for 1 cycle, followed by 30 cycles at 94°C for 1 min (denaturation), 55-65°C for 1 min (annealing), 72°C (extension) for a length determined by product length (~1 min/kb), then one final extension cycle of 72°C for 10 minutes, before being held at 4°C. For viewing, 10 µl of
each 25μl reaction was run out on a 0.8% (w/v) agarose gel with 2μg/ml ethidium bromide and visualized using a MultiDoc-It Digital Imaging System (UVP).

2.6 Sequencing

PCR products were purified from an agarose gel slice with a GFX™ PCR DNA & Gel Band Purification kit (GE Healthcare) according to the manufacturer’s protocol. Constructs were purified from overnight starter cultures grown in Luria Broth (LB) media with antibiotic with a QIaprep® Spin Miniprep Kit (Qiagen). The samples were sequenced using primers designed to sequence along the entire insert piece. Samples were sent to NANUQ, the McGill University and Genomic Quebec Innovation Centre (https://genomequebec.mcgill.ca/nanuq).

2.7 Plant RNA extraction

RNA extractions were carried out using a TRIzol® reagent (Invitrogen) as specified by the manufacturer. Briefly, equipment was washed and treated with RNaseZap® (Ambion) to decrease RNase contamination and its associated possibility of RNA degradation. Approximately 500mg of flower bud, leaf, or root tissue samples were ground to fine powder using liquid nitrogen-chilled mortar and pestle. Once ground, 3mL of TRIzol® was added and the sample was divided into 3 tubes and incubated at room temperature for 5 minutes and transferred to a fresh sterile tube. The samples were centrifuged for 10 minutes at 9000 rpm at 4°C. The overlaying clear phase was transferred to a clean sterile tube and 0.2ml of chloroform was added, the tubes were vigorously shaken for 15 seconds, incubated at room temperature for 3 minutes and then centrifuged for 15 minutes at 9000 rpm at 4°C. Following this, the colourless upper aqueous phase was transferred to a clean sterile tube containing 0.5ml of isopropanol and mixed. The samples were incubated at room temperature for 10 minutes and then centrifuged for 15 minutes at 4°C. The pellet was washed with 75% EtOH (made with DEPC (diethylpyrocarbonate)-treated water) and mixed with vortexing then centrifuged at 4000 rpm for 5 minutes at 4°C. The RNA pellet was air-dried for 20 minutes and RNA was resuspended with DEPC-treated water, chilled on ice for 1 hour and then placed at 60°C for 10 minutes. RNA samples were stored at -80°C.
2.8 cDNA synthesis and expression screening RT-PCR

Because both *AtPUB18* and *AtPUB19* lack any introns, removal of any residual DNA prior to reverse transcription was imperative to distinguish RNA/cDNA amplification from that of gDNA. DNase treatment was carried out using DNase I (Invitrogen) as specified by the manufacturer. Briefly, for each 1μg of RNA sample, 1x DNase I reaction buffer and 1 unit of DNase I (Amplification grade) was mixed on ice. After a 15-minute incubation at room temperature, the DNase I in the mixture was inactivated by adding 2.27mM (25nmol) EDTA solution and incubating at 65°C for 10 minutes.

First strand cDNA synthesis was carried out using SuperScript™ II Reverse Transcriptase (Invitrogen) as specified by the manufacturer. Briefly, 450ng of DNA-free total RNA was mixed with 41.7μg/ml (500ng) of oligo(dT)_{12-18} and 0.83mM (10nmol) dNTPs and heated to 65°C for 5 minutes and chilled on ice for 2 minutes. To this mixture, 1.05x first-strand buffer, 10.5mM (200nmol) DTT, and 40 units of RNaseOUT™ were added and a 42°C incubation occurred for 2 minutes. Each sample was then divided into two fresh sterile tubes. To one tube (+RT), 200 units of SuperScript™ II RT was added and to the other (-RT), an equal volume of DEPC-treated water was added, the latter serving as a control against genomic DNA contamination. All samples were incubated at 42°C for 50 minutes before the reaction was inactivated by heating at 70°C for 15 minutes. The cDNA was ready to be directly used as a template for PCR amplification. Screening of expression via this RT-PCR amplification utilized the same gene-specific set of primers as those used for genotypic screening PCR reactions. Storage of cDNA occurred at -20°C.

2.9 Microscopy

2.9.1 Dissection of siliques

Developmental analysis of non-desiccated siliques was done through the dissection of one of the two locules under a dissecting microscope. Fine-tipped tweezers were used to peel back and remove the pre-dehiscent valve starting at the basal margin at the gynophore/replum. Viable seeds/non-viable ovules were observed under a stereomicroscope (Leica MZ16, Microsystems). Images were taken with Leica Application Suite (v2.5.0).
2.9.2 *Alexander staining of pollen*

To distinguish between viable and non-viable pollen, anthers ranging in age from near-dehiscence to dehiscing found in mature and freshly-opened flowers were placed in a drop of Alexander’s stain (Alexander, 1969). The Alexander staining solution was composed of 10ml 95% ethanol, 10mg malachite green (1ml of 1% solution in 95% ethanol), 50ml dH2O, 25ml glycerol, 5g phenol, 50mg acid fuchsin (5ml of 1% solution in water), 5mg orange G (0.5ml of 1% solution in water), 2ml glacial acetic acid (Johnson-Brousseau and McCormick, 2004). A coverslip was placed on the anthers and gentle pressure was applied to allow stain penetration into the anthers. Slides were observed under an Olympus BX50W1 upright light microscope equipped with a MicroPublisher digital camera from QImaging. Images were taken with QCapture Software (v2.7).

2.10 *Generation of double mutant lines and backcrosses*

For the generation of double mutant lines, a flower with dehisced anthers from one T-DNA line in one gene (in homozygous form) was used to hand-pollinate the emasculated flower of one T-DNA line in the second gene (also in homozygous form). The pollinated pistils were then covered with plastic wrap and left for 24 hours, the wrap removed and the silique allowed to develop. The seeds of the cross (F1 generation) were harvested, sterilized and grown on soil. The plants grown from the F1 generation seed were PCR-genotyped to ensure that T-DNA insertions from both parental crossing lines were present (in double heterozygous form). To generate double homozygous mutant lines, these plants were allowed to self-fertilize and plants grown from the subsequent seeds (F2 generation) were PCR-genotyped to identify mutant lines with a homozygous mutation in both genes. In the event that no double homozygous progeny were found, plants showing homozygosity for one gene and heterozygosity for the other gene were allowed to self-fertilize for better odds of generating double homozygous progeny.

For the generation of the backcrosses, several hand-pollinations were performed as above between the plants of interest. The backcrosses were performed in reciprocal. The seeds of the backcrosses were collected, sterilized, and grown on agar plates.
Each individual progeny seedling was PCR-genotyped and observed ratios from the backcrosses were assessed.

2.11 Generation of promoter DEX::PUB18 transgenic lines

For the generation of the DEX promoter::PUB18 gene construct, the PUB18 gene was amplified from wild-type gDNA, which was possible because this gene comprises one sole exon. A 2113bp XhoI/XhoI fragment was amplified using the following primers 5'-gcgCTCGAGAATGATCCATACGAAAACC GGGTCT-3' and 5'-gcgCTCGAGTCA ACCAGCGTGAACGAA CCGGTC-3' (restriction sites are underlined; start and stop codons are bolded) and cloned into pCR®2.1-TOPO® (Invitrogen). After verification by DNA sequencing, the insert was cloned just downstream of the inducible promoter using the XhoI site in the two-component glucocorticoid-inducible vector pTA7001 a binary GVG transformation vector harbouring the trans-element under the control of the 35S promoter (Aoyama and Chua, 1997). The vector was introduced into plants through an Agrobacterium tumefaciens-mediated (strain GV3101) floral dip transformation method (Clough and Bent, 1998). Transformants were screened by hygromycin selection (0.05mg/ml) and confirmed with genotype screening to be carrying the DEX::AtPUB18 construct with the following primers 5'-ACCCTTCCTCTCTATATAAGGAAGT-3' (vector sequence) and 5'-CCAGACAAAGACCTAT AAAAACTG-3' (gene sequence).

2.11.1 DEX-driven expression as a rescue attempt

For use in the attempt to rescue gametophytic development, the vector with the DEX promoter::PUB18 gene construct was introduced into double heterozygous plants (pub18-1/+ pub19-1/+). Induction was done using dexamethasone (DEX; Bioshop), a strong glucocorticoid derivative, which was first dissolved in ethanol as a 30mM stock before being diluted into a spraying solution (30µM, 0.01% (v/v) Tween-20 (BioShop)). This DEX solution was sprayed every other day onto flowering plants, focusing particularly on unopened floral buds. For use for any future complementation assays, the vector was also introduced into double homozygous plants (pub18-1 pub19-1, pub18-3 pub19-3), single homozygous plants (pub18-1, pub18-3, pub19-1, pub19-3),
and wild-type. For control purposes, the empty vector pTA7001 was also transformed into wild-type plants.

2.12 Germination assays

Seed from wild-type and transgenic *Arabidopsis* plants grown simultaneously were harvested at the same time and stored under the same conditions. For proper comparison purposes, seeds originating from the same batch were used for germination and root growth assays. Wild-type and transgenic *Arabidopsis* seeds were surface sterilized and placed on media plates containing 0.5x Murashige and Skoog (MS) salts, 1% (w/v) sucrose, 0.8% (w/v) phytoagar, pH 5.7 and any additional treatment compound. For ABA treatment, a fresh 20mM stock of ABA (Sigma) dissolved in 100% methanol was made and added to post-autoclaved media for various final concentrations (1-10μM); an equal volume of 100% methanol to that added for the highest ABA concentration was used in control plates. For salt stress testing, NaCl was added to media for various final concentrations (100-150mM) prior to autoclaving. After sterilization and appropriate chemical addition, cooled media (< 60°C) was aliquotted into Petri dishes for even solidification. After 3 days of stratification, the plates were placed under long-day photoperiod (16hr light / 8hr dark cycle) at 22°C in a growth chamber (Percival Scientific). With the use of a dissecting scope (Leica), seeds were scored as germinated upon radicle emergence from the seed coat. Germination was scored daily for 4-10 days. All data are presented as mean ± standard error of three replicates (n = 48).

2.13 Root length assays

Wild-type and transgenic *Arabidopsis* seeds were surface sterilized and placed on media plates containing 0.5x Murashige and Skoog (MS) salts, 1% (w/v) sucrose, 0.8% (w/v) phytoagar, pH 5.7. After 3-4 days of stratification, the plates were vertically oriented and seedlings were allowed to grow under long-day photoperiod (16hr light / 8hr dark cycle) at 22°C in a growth chamber (Percival Scientific). After 3 days of incubation, seedlings of similar size and primary root growth length were transferred to fresh plates with media supplemented with various abiotic stress-inducing compounds and further vertical growth was monitored over the course of one week. After
sterilization and appropriate chemical addition, cooled media was equally aliquotted into Petri dishes for even solidification.

For ABA treatment, a fresh 20mM stock of ABA (Sigma) dissolved in 100% methanol was made and added to post-autoclaved media for various final concentrations (0.1-10μM); an equal volume of 100% methanol to that added for the highest ABA concentration was used in control plates. For salt stress testing, NaCl (BioShop) was added to media for various final concentrations (50-150mM) prior to autoclaving. For osmotic stress testing, D-mannitol (BioShop) was added to media for various final concentrations (150-300mM) prior to autoclaving. For oxidative stress testing, a 19.4mM (5mg/mL) stock of methyl viologen (also known as paraquat, Aldrich) was dissolved in dH$_2$O and filter-sterilized before being added to post-autoclaved media for various final concentrations (0.2-1.5μM). For heat stress treatment, a 56.8μM (10mg/mL) stock of L-canavanine (Sigma) was dissolved in dH$_2$O and filter-sterilized before being added to post-autoclaved media for various final concentrations (1-8μM). For ABA-inhibition testing, a fresh 50mM stock of fluridone (also known as Sonar, Chem Service) dissolved in 100% methanol was made and added to post-autoclaved media for various final concentrations (1-100μM); an equal volume of 100% methanol to that added for the highest fluridone concentration was used in control plates. While all of the above root assays were performed in a growth chamber at 22°C under long-day conditions at regular-light levels (90-100μE s$^{-1}$ m$^{-2}$; Percival Scientific), the combination of salt and high-light stress testing also utilized a growth chamber at 22°C under long-day conditions at high-light levels (300-350μE s$^{-1}$ m$^{-2}$; Percival Scientific). After 3 days of initial growth on salt-free culture media under regular- or high-light, seedlings were transferred to salt plates (see above) and placed back under regular- or high-light conditions or exchanged chambers to change light conditions.

Images were taken immediately after seedling transfer and after 3, 5, and 7 days of additional growth (Canon PowerShot S60 digital camera). Primary root axis lengths were measured from digital photographs using ImageJ software. Relative root length growth was determined by dividing average length of additional growth with treatment
by average length of additional growth without treatment (ie. MS only or control). All data are presented as mean ± standard error of three replicates (n = 6).

### 2.14 Chlorophyll retention assay

For leaf senescence induction, the leaves of four-week-old wild-type and transgenic *Arabidopsis* vegetative rosettes were detached and floated on dH₂O in small Petri dishes for three and seven days in permanent darkness at ambient temperature. For each genotype, three leaves per day were measured for their chlorophyll content. Each leaf sample was ground with a pestle after cooling in liquid nitrogen and homogenized in an extraction buffer. Chlorophyll was extracted with a buffered acetone extraction solution (80% acetone, 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, BioShop), pH 7.5). After centrifugation (3min at 14,000rpm), light absorbance of the extract was recorded at 646 and 663nm with a UV/Vis spectrophotometer (Ultraspec 2100 pro), and the total chlorophyll content was calculated with $17.76A_{646} + 7.34A_{663}$ and normalized to original fresh weight as previously described (Porra *et al.*, 1989). As a reference, the total chlorophyll content at the start of the experiments was determined.
CHAPTER 3: RESULTS

3 Results

3.1 AtPUB18 and AtPUB19 – two closely related genes with shared expression patterns upon abiotic stress treatments

Previously, 17 PUB family members in Arabidopsis that contained both an ARM repeat domain downstream of the U-box and a UND upstream of the U-box had been identified (Mudgil et al., 2004). Four of these PUB-ARMs encode a plant U-box protein without any introns, AtPUB16 (At5g01830), AtPUB17 (At1g29340), AtPUB18 (At1g10560), and AtPUB19 (At1g60190). Of these four, AtPUB19 is notable for its high up-regulated expression upon abiotic stresses such as cold, osmotic, and salt stresses in both root and shoot tissues and, when considering all 64 PUBs, in fact demonstrates the strongest up-regulation in the microarray expression data sets for these treatments (Figure 2B, 2C; Figure 3A; Yee and Goring, 2009). Similarly, with respect to the regulation of expression by plant hormones, in particular ABA, AtPUB19 exhibited the strongest induction when considering the entire PUB gene family (Figure 2A; Figure 3C; Yee and Goring, 2009). AtPUB18 and AtPUB19 have highly similar expression profiles, as assessed by the average linkage hierarchical clustering and Pearson correlation coefficients (Figure 3A). More specifically, up-regulation of AtPUB18 is also consistently seen with these same treatments (cold, osmotic, salt, ABA), albeit at much lower levels.

AtPUB18 and AtPUB19 share 64% identity and 95% similarity in amino acid sequence (Figure 4C). Previously, AtPUB18 was also found to have E3 ubiquitin ligase activity through an in vitro assay (Mudgil et al., 2004). A phylogeny was generated based on ARM repeat domain sequence similarity between the 17 members of the UND-containing AtPUB-ARM family and in accordance with previously published phylogenetic analyses of the AtPUB-ARMS based on the U-box domain and the UND (Mudgil et al., 2004), AtPUB19 formed a consistently strong clade with AtPUB18, which, as with other analyses, also grouped at a comparatively greater genetic distance from other clusters (Figure 5). This relatively conserved AtPUB18 / AtPUB19 clade supports the strong possibility that AtPUB18 and AtPUB19 may work redundantly.
Figure 3: Gene expression microarray profiles for the 17-UND containing AtPUB-ARM genes in Arabidopsis across a range of conditions and treatments.

The database AGI gene identifiers are listed alongside the AtPUB gene names. *AtPUB18* and *AtPUB19* are highlighted. *AtPUB4* (*At2g23140*) is not present in the microarray database. Electronic-Northern heat maps were generated from results collected from the public *AtGenExpress Consortium* data sets (Kilian *et al.*, 2007; Goda *et al.*, 2008) accessible through the Expression Browser tool (Toufighi *et al.*, 2005) and resubmitted into DataMetaFormatter genomic tool, both found at BAR ([www.bar.utoronto.ca](http://www.bar.utoronto.ca)). The colour scale indicates the log2-level of expression above (red) or below (blue) the median. Co-expression at the level of expression between genes can be determined by the average linkage hierarchical clustering and the Pearson correlation coefficient as indicated by the respective tree and scale to the right of the gene names.

A) *AtPUB* expression profiles from wild-type seedlings in response to abiotic stress treatments with co-expression seen between *AtPUB18* and *AtPUB19*. Shoot and root tissues from 18-day-old plants were analyzed after 0.5, 1, 3, 6, 12, and 24 hours of treatment (each block a time point from left to right). For cold stress, plants were moved into 4°C. For osmotic stress, 300mM mannitol was added to the growth media. For salt stress, 150mM NaCl was added to the growth media. For drought stress, plants were removed from liquid media and subjected to the air stream for 15 minutes, resulting in approximately 10% loss in fresh weight. Data were obtained from the AtGenExpress Abiotic Stress Series (Kilian *et al.*, 2007) hosted at BAR.

B) *AtPUB* expression profiles from wild-type seedlings in response to abiotic stress treatments without co-expression seen between *AtPUB18* and *AtPUB19*. Shoot and root tissues from 18-day-old long-day-grown plants were analyzed after 0.5, 1, 3, 6, 12, and 24 hours of treatment (each block a time point from left to right). For oxidative stress, 10μM of methyl viologen was added to the growth media. For UV-B light stress, plants were subjected to UV-B light for 15 minutes. For heat stress, plants were placed
at 38°C for 3 hours and allowed to recover at 25°C. Data were obtained from the AtGenExpress Abiotic Stress Series (Kilian et al., 2007) hosted at BAR.

C) *AtPUB* expression profiles from wild-type seedlings and seeds in response to ABA hormone treatment. For seedlings, 7-day-old continuous light-grown plants were analyzed 0.5, 1, and 3 hours after treatment with 10μM of ABA (each block a time point from left to right). Data were obtained from the AtGenExpress Hormone Series (Goda et al., 2008). For seeds, 3μM and 30μM ABA were used to treat 2-to-4-month-after-ripened samples and analysis followed after 24 hours of imbibition. Data provided from the Nambara Lab (http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=183). All data hosted at BAR.
Figure 4: Sequence analysis and localization of T-DNA insertion sites for mutant lines for AtPUB18 and AtPUB19.

Schematic illustration of each gene of interest, each comprises a sole exon, with the T-DNA insertion shown as inverted triangles. The conserved U-box domain is shown, as determined by Pfam. Bar = 500bp.

A) Gene structure of AtPUB18 (At1g10560)’s 2094 base pairs showing the positions of the T-DNA insertions for pub18-1 (SAIL_634_G01) at nucleotide 822, pub18-2 (SALK_001831) at nucleotide 2008, pub18-3 (GABI-Kat_428E05) at nucleotide 1417.

B) Gene structure of AtPUB19 (At1g60190)’s 2061 base pairs showing the positions of the T-DNA insertions for pub19-1 (SALK_152677) at nucleotide 1563, pub19-2 (SALK_058791) at nucleotide 2056, pub19-3 (GABI-Kat_099D10) at nucleotide 1190.

Gene-specific and T-DNA-specific primers used in the PCR-based genotyping and RT-PCR are listed in Appendix III.

C) Protein sequence alignment of AtPUB18 and AtPUB19 showing 64% identity and 95% similarity. Asterisk (*) denotes identical residues at that position, colon (:) indicates residues at that position are very similar, dot (.) indicates residues at that position are more or less similar, and green lettering with no markings denotes residues with no common properties.
UND ARM repeats U-box UTR

AtPUB18 MIHTGGSRILTFTPTVPEPSESISITVLJLSQILADGSLITFTKSHFSTNKQSFRETLRQRNLLVVFEEIREIR
AtPUB19 MIHTGGSRILTFTFANPCESISITTLVSDQIQAGEILSFPPKHFSTNKQSKVLFTLHVQTVLIPREELIQ

AtPUB18 IRNRSRVPYDSAlAASLSKEHIFVQKLFPLLEDTRQGARLCM0MNSDQVSDNLRLVRTRSIESTLQAFPVASVDL
AtPUB19 IRVGSIPAGRSVILS-LSELHIFVQKLFPLLEDTRQGARLCM0MNSQVSAHFEDRLTRSIESTLQFVVRSDL

AtPUB18 TTEVNELIDLVVRQARKYGVQPETNDKRAVSSINRILALFVVRVVPDPDEINRILDHVGIRKWLDCVKEINFLGE
AtPUB19 PGEVNELIYLVMRQTRKSEARPDRDDKRAIDSVLYFTVFVVRVVPDVNSDEILRVLDHVGIRKWLDCVKEINFLRE

AtPUB18 EIDAERLDEKKKKSSDQVELLSLMGFICRYCRCILIGRIERDDHHNHDGIKKDHDLIRGLKVEDLLCPISLEI
AtPUB19 EISVGL------KKSNIEIELLSLMGFICRYCRCILIGIDVDDEEKDKEEDDLKIVRSLNVDLDCPISLEI

AtPUB18 MTFVIEBGTHTDSDITSITKBEQGKMSLQTVQYVEIRQKTCGKILSTSLVDNVSVRQVRKHHCKTGNYGLASERLRKSHDDV
AtPUB19 MTFVIEBGTHTDSDITSITKBEQGKMSLQTVQYVEIRQKTCGKILSTSLVDNVSVRQVRKHHCKTGNYGLASERLRKSHDDV

AtPUB18 VPESLAAGKGLITAFLSSELINGGEEFYAYAVRIYVSTKSSFPRSLCVKAGAVTPPLKLILLSDVRIKENA
AtPUB19 VAESLAAGKGLITAFLSSELINGGEEFYAYAVRIYVSTKSSFPRSLCVKAGAVTPPLKLILLSDVRIKENA

AtPUB18 MAGILNLSKHTGSSKIAGE---GLKMLVIELNEMGAKTTRLYASALFYLSELLVEDYSLRIGENPDACIPGLMNIV
AtPUB19 MAGIMNLSKDIAGKLVISOGEDGGLRLIVEVLNDGARRESQYAAAALFYLSSLGDYSLRIGENPDACIPGLMNIV

AtPUB18 KGDDYGSASKKSALEAVMGFSMDSNWRVYLAAGAPFILLLLRLSNEGILSGGLTADCATLAAKAYEFDGTIGVI
AtPUB19 KGDDYGSASKKSALEAVMGFSMDSNWRVYLAAGAPFILLLLRLSNEGILSGGLTADCATLAAKAYEFDGTIGVI

AtPUB18 RRGRLAKVIISSSDEPSDVAVKQCTVGLILNLCLNGDVRGVLWNLNHSMWGLSUTTVSNSSQYGGSGKASLIR
AtPUB19 RRGRLAKVIISSSDEPSDVAVKQCTVGLILNLCLNGDVRGVLWNLNHSMWGLSUTTVSNSSQYGGSGKASLIR

AtPUB18 MIHEFQERTGSVEPNLQRGRFVHAW
AtPUB19 MIHEFQERTGSVEPNLQRGRFVHAW
Figure 5: Phylogenetic relationships of select plant U-box proteins in Arabidopsis.

A) Phylogeny of U-box sequences from the 41 AtPUB-ARM proteins (modified from Mudgil et al., 2004).

B) Phylogeny of UND domain sequences from the 17 AtPUB-ARM proteins with the UND (modified from Mudgil et al., 2004).

C) Domain organization of a typical UND-containing AtPUB-ARM protein. UND – U-box N-terminal domain; U-box – UFD2 homology; ARM – Armadillo repeat (from Mudgil et al., 2004).

D) Phylogeny of ARM repeat domain sequences from the 17 AtPUB-ARM proteins with the UND

The database AGI gene identifiers were listed alongside the AtPUB gene names. AtPUB18 and AtPUB19 are highlighted.
Based on publicly-available data from microarray expression data sets, the expression of *AtPUB18* and *AtPUB19* during general plant development could be readily illustrated (Figure 6; Winter *et al.*, 2007). Overall, consistent with the conditional expression data sets, *AtPUB18* expression was lower than *AtPUB19* expression but shared many same localizations. For example, expression of both genes could be seen in roots from 17-day-old wild-type seedlings, and to a lesser extent from 7-day-old seedlings (Figure 6B; Schmid *et al.*, 2005); increasing expression could be seen in the central zone, peripheral zone, to the rib meristem of the shoot apical meristem (SAM) of *ap1-1 cal1-1* (a double mutant that converts floral meristems to shoot meristems) (Figure 6F; Yadav *et al.*, 2005). However, there were also some contrasts in gene expression patterns. For example, while expression of both genes could be seen in the sepals, petals, and stamens of stage 12 flowers, by stage 15 flowers, expression only remained in the sepals for *AtPUB19* and in the petals for *AtPUB18* (Figure 6A; Schmid *et al.*, 2005); while expression was clear in all the root cell types (lateral root cap, epidermis, epidermal artrichoblasts, endodermis (+ cortex), stele), gene levels increased from stage I to stage II to stage III cells for *AtPUB19*, levels increased from stage III to stage II to stage I (with the highest expression in the quiescent center) for *AtPUB18* (Figure 6G; Birnbaum *et al.*, 2003; Nawy *et al.*, 2005); in a comparison between guard and mesophyll cells expression of *AtPUB18* was higher in mesophyll, while expression of *AtPUB19* was higher in guard cells (Figure 6H; Yang *et al.*, 2008). And in a further illustration of increased *AtPUB19* levels upon ABA treatment, this latter example also shows up-regulation in both the mesophyll and the guard cells upon spraying with a solution containing ABA (Yang *et al.*, 2008). And lastly, expression of *AtPUB19* could be identified where no notable detection of *AtPUB18* occurred. For examples, in the senescing leaf, cauline leaf (Figure 6D), stage 4 seeds with siliques (Figure 6C), and in dry and imbibed seeds (Figure 6E; Schmid *et al.*, 2005; Nambara lab).

### 3.2 Isolation and characterization of *pub18* and *pub19* T-DNA insertion lines

To study the function of *AtPUB18* and *AtPUB19* in plant development, three insertion mutants for each gene (renamed *pub18-1*, *pub18-2*, *pub18-3* for *AtPUB18*; renamed *pub19-1*, *pub19-2*, *pub19-3* for *AtPUB19*) from various T-DNA collections (see Materials
**Figure 6: Expression patterns of AtPUB18 and AtPUB19 during various stages and tissues of plant development.**

The schematic representations of absolute expression from the Development Series data set were generated with the *Arabidopsis eFP* Browser at BAR ([www.bar.utoronto.ca](http://bar.utoronto.ca)).

**A)** Stage 12 (top) and stage 15 (bottom) flowers showing expression levels for sepals, petals, stamens, and carpels (Schmid *et al.*, 2005)

**B)** 7-day-old (left) and 17-day-old (right) seedlings showing expression levels for cotyledons, hypocotyl, and roots of the former and vegetative rosette and roots of the latter (Schmid *et al.*, 2005)

**C)** Siliques (top) with stage 4 seeds (bottom) (Schmid *et al.*, 2005)

**D)** Cauline (left) and senescent (right) leaves (Schmid *et al.*, 2005)

**E)** Dry (top) and 24-hr-imbibed (bottom) seed (Nambara lab [http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=183](http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=183))

**F)** Central zone (top), rib meristem (middle), and peripheral zone (bottom) of the shoot apical meristem (SAM) of *ap1-1 cal1-1* (a double mutant that converts floral meristems to shoot meristems) (Yadav *et al.*, 2009)

**G)** Root cell type expression from the quiescent center, and the stele, endodermis, cortex, epidermal arichoblasts, epidermis (II, III), and lateral root cap (I, II) (cell types from center out to periphery) of stage I, II, III roots (stages increasing from tip upwards) (Birnbaum *et al.*, 2003; Nawy *et al.*, 2005)

**H)** Leaf cross sections showing expression in mesophyll and guard cells after control (top) and ABA treatment (bottom) (Yang *et al.*, 2008)
and Methods) were isolated. Using PCR genotyping, plants homozygous for the T-DNA insertion (potential knock-outs) and wild-type to the T-DNA insertion (non-transgenic siblings) were established from segregating lines. To confirm the location of the T-DNA insert within the gene of interest, sequence analysis of the region flanking the T-DNA was performed (Figure 4A). The T-DNA inserts for *pub18-1* (SAIL_634_G01) are oriented in both the forward and reverse directions and is located at the 822bp position of the sole exon of the 2094bp *AtPUB18* gene. The T-DNA inserts for *pub18-2* (SALK_001831) are also oriented in both forward and reverse directions and is located at the 2008bp position. The T-DNA insert for *pub18-3* (GABI-Kat_428E05) is oriented in forward direction, 1417bp downstream from the start codon. The T-DNA insert for *pub19-1* (SALK_152677) is oriented in the reverse direction and is located at the 1563bp position of the sole exon of the 2061bp *AtPUB19* gene. The T-DNA insert for *pub19-2* (SALK_058791) is oriented in reverse, 2056bp after the start codon. The T-DNA insert for *pub19-3* (GABI-Kat_099D10) is oriented in forward direction, at the 1190bp position.

To validate the knock-out status of the plant lines in use for this project, I extracted total RNA from tissue shown to highly express *AtPUB18* or *AtPUB19* (Figure 6). RNA was extracted from floral buds for *AtPUB18* gene expression detection in *pub18-1* and *pub18-2*, from leaves for *AtPUB19* gene expression in *pub19-1* and *pub19-2*, and from roots of vertically-grown seedlings in *pub18-3* and *pub19-3* mutants. RT-PCR analyses successfully showed no transcripts (i.e. no band) when amplification was directed to flank the T-DNA insertion location for *pub18-1*, *pub18-3*, *pub19-1*, *pub19-2*, and *pub19-3* mutants (Figure 7). This shows that across the insertion site, these T-DNA lines are nulls, thus validating these lines as gene knock-outs. Because RT-PCR analysis of *pub18-2* failed to show complete loss of amplification across the T-DNA insertion site, further 5’end-specific reactions were performed on this line, as well as on *pub19-2*. These two T-DNA insertion lines have a T-DNA insertion site extremely close to the 3’end of their respective genes (Figure 7B, 7E) and perhaps because the T-DNA insertion site is located so close the 3’end of the gene, it did not appear to greatly affect gene expression (as assessed by 5’end reactions). As such experiments and analyses performed on the *pub18-2* and *pub19-2* were minimized to avoid focusing on lines that
Figure 7: RT-PCR analysis of wild-type and transgenic lines.

Independent homozygous T-DNA insertion lines were analyzed for \textit{AtPUB18} gene expression (left column) or for \textit{AtPUB19} gene expression (right column) from total RNA extracted from tissue shown to highly express \textit{AtPUB18} or \textit{AtPUB19}.

A) \textit{pub18-1} – RNA was extracted from floral buds

B) \textit{pub18-2} – RNA was extracted from floral buds

C) \textit{pub18-3} – RNA was extracted from roots

D) \textit{pub19-1} – RNA was extracted from leaves

E) \textit{pub19-2} – RNA was extracted from leaves

F) \textit{pub19-3} – RNA was extracted from roots

As a positive control, transcripts extracted from respective non-transgenic sibling plants were also amplified. As a loading control, the transcript level of \textit{actin3} was also simultaneously assessed.

To optimize RT-PCR reaction, specific primers were designed to flank the gene position (Figure 4) of each T-DNA insertion. Because of the insertion position of \textit{pub18-2} (B) and \textit{pub19-2} (E) being so close to the 3’end of the \textit{AtPUB18} and \textit{AtPUB19} gene, respectively, an additional reaction amplifying for the 5’end of each gene was included for these T-DNA mutants.

While every flanking reaction showed an attenuation of gene expression, reactions for the 5’end were positive, thus justifying the elimination of \textit{pub18-2} and \textit{pub19-2} from further use because of the likelihood of these lines representing non-null transgenic plants.
were not guaranteed to be nulls. Thus, in an attempt to be more assured of full knock-outs, the location of the T-DNA insertion was taken into consideration when selecting lines most suitable for gene knock-out studies. Overall, plants homozygous for the T-DNA insertion and non-transgenic siblings were grown simultaneously to bulk up seeds in preparation for comparison during germination and root length assays (see below).

3.3 \textit{pub18} and \textit{pub19} mutants show no phenotypic differences during general plant growth and development

When grown on soil, homozygous \textit{pub18-1}, \textit{pub18-3}, \textit{pub19-1}, and \textit{pub19-3} mutants exhibited no readily discernible phenotypes when compared to wild-type plants under normal growth conditions. Leaves from three week old plants, rosettes from four week old plants, and mature flowering six week old plants of single homozygous lines all appeared to have comparable growth and development to that of wild-type plants (Figure 8A, 8C, 8D, 8F, 8H; Figure 9B, 9C, 9E, 9F). Similarly, health and appearance of seeds in greening siliques and of pollen of mature anthers also appeared normal in these lines (Figure 10E, 10F; Figure 11E, 11F). Overall, phenotypic analysis of general plant development did not yield any obvious distinct differences between wild-type and single knock-out \textit{AtPUB18} and \textit{AtPUB19} T-DNA lines. Having confirmed that these T-DNA insertion lines are indeed knocking-out \textit{AtPUB18} or \textit{AtPUB19} expression, and given high similarity in sequence identity (Figure 4C) and similar expression patterns (Figure 6), the strong probability that closely related AtPUB-ARMs are at least partially functionally redundant might account for the lack of phenotypic difference observed between single homozygous mutant and wild-type plants.

3.4 The generation of an initial set of double knock-out lines show an increase in pollen collapse and ovule abortion in plants heterozygous for both genes

To alleviate the scenario that \textit{AtPUB18} and \textit{AtPUB19} might have functional redundancy, an initial attempt was made to generate double knock-out mutants in both \textit{AtPUB18} and \textit{AtPUB19} genes with select T-DNA insertion mutants previously identified as nulls. Specifically, reciprocal crosses were made between \textit{pub18-1} and \textit{pub19-1} knock-out lines. No readily discernible differences in plant growth were seen in the F1
Figure 8: Developmental phenotypes of non-flowering wild-type and transgenic mutants under normal growth conditions.

A) Morphological leaf series comparisons of representative 3-week-old wild-type, *pub18-1 pub19-1*, *pub18-3 pub19-3*, *pub18-1, pub19-1, pub18-3*, and *pub19-3* (in order, from top to bottom) plants grown in soil. Bar = 1cm.

Morphological vegetative rosette comparisons of representative 4-week-old plants:

B) wild-type

C) *pub18-1*

D) *pub19-1*

E) *pub18-1 pub19-1*

F) *pub18-3*

G) *pub19-3*

H) *pub18-3 pub19-3*

Plants grown in soil under long-day conditions (16hr light / 8 hr dark photoperiod). Bar = 1cm.
Figure 9: Developmental phenotypes of flowering wild-type and transgenic mutants under normal growth conditions.

Morphological whole plant comparisons of representative 6-week-old plants:

A) wild-type

B) pub18-1

C) pub19-1

D) pub18-1 pub19-1

E) pub18-3

F) pub19-3

G) pub18-3 pub19-3

Plants grown in soil under long-day conditions (16hr light / 8 hr dark photoperiod). Bar = 3cm.
A wild-type  

B pub18-1  

c pub18-3  

C pub19-1  

D pub18-1 pub19-1  

E pub18-3 pub19-3  

F pub19-3  

G pub18-3 pub19-3
Figure 10: Ovule morphology of wild-type and transgenic silique.

Mature, non-desiccated representative silique from following plants:

A) wild-type

B) pub18-1 pub19-1

C) pub18-3 pub19-3

D) pub18-1/+ pub19-1/+  

E) pub18-1

F) pub19-1

Plants were dissected to reveal green, normally developing seeds with healthy embryos. Bar = 1mm.

While silique from the seemingly non-independently-assorting double heterozygous pub18-1/+ pub19-1/+ initially showed a proportion of shriveled ovules (red arrows), silique from single homozygous pub18-1 and pub19-1 did not show an equivalent ovule abortion. Eventually, double homozygous pub18-1 pub19-1 plants were generated and these revealed no shriveled ovules to the extent observed in pub18-1/+ pub19-1/+ plants. Silique dissection of subsequent pub18-3 pub19-3 plants confirmed that the ovule abortion seen in pub18-1/+ pub19-1/+ was not related to mutations in the AtPUB18 or AtPUB19 genes.
**A** wild-type

**B** pub18-1 pub19-1

**C** pub18-3 pub19-3

**D** pub18-1/+ pub19-1/+ [red arrows indicating a difference]

**E** pub18-1

**F** pub19-1
Figure 11: Pollen phenotypes of wild-type and transgenic anthers.

Dehiscing anthers from mature flowers of following plants:

A) wild-type

B) \textit{pub18-1 pub19-1}

C) \textit{pub18-3 pub19-3}

D) \textit{pub18-1/+ pub19-1/+}

E) \textit{pub18-1}

F) \textit{pub19-1}

Anthers were stained with Alexander staining to assess pollen viability. Bar = 0.1mm.

Viable pollen grains are circular in shape and stain a pink/purple, while non-viable pollen grains appear as collapsed structures that stain a turquoise blue/green. While pollen from the seemingly non-independently-assorting double heterozygous \textit{pub18-1/+ pub19-1/+} initially showed a proportion of collapse, pollen from single homozygous \textit{pub18-1} and \textit{pub19-1} did not show an equivalent non-viability. Eventually, double homozygous \textit{pub18-1 pub19-1} plants were generated and these revealed no collapsed pollen grains as extensive as that found in \textit{pub18-1/+ pub19-1/+} plants. Anther staining of subsequent \textit{pub18-3 pub19-3} plants confirmed that the pollen non-viability seen in \textit{pub18-1/+ pub19-1/+} was not related to mutations in the \textit{AtPUB18} or \textit{AtPUB19} genes.
A wild-type

B pub18-1 pub19-1

C pub18-3 pub19-3

D pub18-1/+ pub19-1/+  

E pub18-1

F pub19-1
generation (all *pub18-1/+ pub19-1/+*) or in the F2 generation (presumably mixed). However, a closer look at the F2 generation yielded interesting results. Dissecting siliques from double heterozygous plants (*pub18-1/+ pub19-1/+*) showed a proportion of abortion, which appeared as small, desiccated structures, while dissected siliques from *pub18-1* and *pub19-1* plants showed only healthy embryos growing inside normal plump green seeds (Figure 10B, 10D). Similarly, Alexander staining, which contains cellulose-staining malachite green and protoplasm-staining acid fuchsin, was used to assess pollen viability; viable pollen grains are circular in shape and stain a pink/purple, while non-viable pollen grains appear as collapsed structures that stain a turquoise blue/green (Alexander, 1969). Pollen from double heterozygous plants (*pub18-1/+ pub19-1/+*) showed a proportion of collapsed non-viable grains while pollen from *pub18-1* and *pub19-1* plants showed healthy viable grains (Figure 11B, 11D).

A possible consequence of such gametophytic non-viability was reflected upon screening of individual progeny from self-fertilized double heterozygous plants (*pub18-1/+ pub19-1/+*). Using gene specific primers that flanked the T-DNA insertion site for *pub18-1* and *pub19-1* in combination with left border primers within the T-DNA insert, genotypic screening of seedlings following germination in this selfed population was performed and segregation of the expected Mendelian ratio of the various mutant allele combinations was assessed. Screening of over 300 plants showed that double homozygous plants (*pub18-1 pub19-1*) were never recovered (Figure 12A). Since plants with one null homozygous gene and the other gene heterozygous (*pub18-1/+ pub19-1/- or *pub18-1/- pub19-1/+*) were rarely recovered, genotypic screening suggested that gametes missing both the *AtPUB18* and *AtPUB19* genes might not be viable. Accordingly, the abortion seen in dissected siliques (Figure 10D) and the collapse seen in stained anthers (Figure 11D) could represent ovules and pollen grains missing both genes.

3.5 Genotypic analysis of progeny screening from self-fertilized double heterozygous plants (*pub18-1/+ pub19-1/+*) suggests gene linkage

Even when the expected Mendelian segregation ratio was adjusted to account for a presumed non-viability of *pub18 pub19* gametes, the observed ratio of the progeny from
PCR genotypic screening was performed on individual plants. In double heterozygous plants, the observed progeny ratios (Observed) were compared to the genotype ratio expected between two alleles with normally independent assortment (Expected-normal) and to that expected when gametes lacking in both genes are presumed null (Expected-null). In single heterozygous plants, the observed progeny ratios were compared to the expected 1:2:1 Mendelian segregation ratio. Chi-square tests were performed comparing Observed genotype distributions to Mendelian expectations when Normal or for the hypothetical case of Null gametes in the case of two independent loci segregation.

A) Genotype distribution of progeny from a selfed pub18-1/+ pub19-1/+ plant. n = 364
Chi-square test of observed distribution for:
“Expected-normal”: $\chi^2 = 748.2$; for degrees of freedom = 8, $P < 0.05$
“Expected-null”: $\chi^2 = 55.5$; for degrees of freedom = 5, $P < 0.05$

B) Genotype distribution of progeny from a selfed pub18-2/+ pub19-1/+ plant. n = 104
Chi-square test of observed distribution for:
“Expected-normal”: $\chi^2 = 21.7$; for degrees of freedom = 8, $P < 0.05$
“Expected-null”: $\chi^2 = 15.8$; for degrees of freedom = 5, $P < 0.05$

C) Genotype distribution of progeny from a selfed pub18-3/+ pub19-3/+ plant. n = 102
Chi-square test of observed distribution for:
“Expected-normal”: $\chi^2 = 6.0$; for degrees of freedom = 8, $P > 0.05$
“Expected-null”: $\chi^2 = 15.5$; for degrees of freedom = 5, $P < 0.05$

D) Genotype distribution of progeny from a selfed pub18-1/+ plant. n = 120
Chi-square test of observed distribution for single allele:
$\chi^2 = 1.65$; for degrees of freedom = 8, $P > 0.05$

E) Genotype distribution of progeny from a selfed pub19-1/+ plant. n = 121
Chi-square test of observed distribution for single allele:
$\chi^2 = 25.3$; for degrees of freedom = 2, $P < 0.05$

F) Genotype distribution of progeny from a selfed pub19-3/+ plant. n = 144
Chi-square test of observed distribution for single allele:
$\chi^2 = 3.5$; for degrees of freedom = 2, $P > 0.05$
self-pollinated double heterozygous plants \((\text{pub18-1}/+ \text{ pub19-1}/+)\) was still significantly deviated. Specifically there was a segregation distortion towards progeny that were double heterozygous \((\text{pub18-1}/+ \text{ pub19-1}/+)\) or were singly homozygous in only one gene \((\text{pub18-1}/- \text{ PUB19-1}/+ \text{ or PUB18-1}/+ \text{ pub19-1}/-)\) (Figure 12A). What was especially perplexing was why, of over 300 screened progeny, wild-type progeny were never recovered.

Looking at the results themselves, evidence pointed to the genes being linked. With gene linkage, instead of each gene independently assorting and crossing-over to yield every combination expected with Mendelian ratios, genes that are linked are transmitted/inherited together. Gene linkage would not be visible until the F2 generation, when the progeny could be classified as parental or recombinant. Gene linkage is distinguishable by the generalization that original/parental classes greatly outnumber the recombinant classes (Hartwell et al., 2004). With the progeny from a selfed double heterozygous plant \((\text{pub18-1}/+ \text{ pub19-1}/+)\) falling in the parental and recombinant classes at a percentage of 96% and 3%, respectively, the data supported this notion of non-independent assortment (Figure 13A). To further support the lack of independent gene assortment, selected reciprocal back-crosses between double heterozygous plants \((\text{pub18-1}/+ \text{ pub19-1}/+)\) and wild-type or single homozygous plants \((\text{pub18-1} \text{ or pub19-1})\) were performed. The genotype screening results from the generated progeny revealed that the ratios were significantly different from that expected of normal Mendelian segregation but were not significantly different from that expected from gene linkage between \(\text{AtPUB18}\) and \(\text{AtPUB19}\) (Figure 14).

3.6 Given the chromosomal locations of \(\text{AtPUB18}\) and \(\text{AtPUB19}\) and a second cross, gene linkage is highly unlikely

\(\text{PUB18}\) and \(\text{PUB19}\) are both located on \(\text{Arabidopsis'}\) chromosome 1 which could explain why there is a lack of independent gene assortment; however, recombination can still occur when genes are far apart on the same chromosome. Based on the \(\text{Arabidopsis'}\) chromosomal map, the \(\text{AtPUB18}\) and \(\text{AtPUB19}\) genes are 18.7 Mbases apart, which one would reasonably believe could be sufficient to allow independent assortment (Figure 13B). Further support that the recombination frequencies in a selfed
Figure 13: Illustration outlining non-independent gene assortment and gene distribution of AtPUB18 and AtPUB19.

A) After crossing parental plants and allowing the F1 generation to self-fertilize, an indicator of gene linkage between two genes is seen when parental combinations outnumber recombinant combinations among the F2 progeny. In comparison, when the numbers of parental and recombinant types of progeny are equal, this indicated independent gene assortment. Selfed double heterozygous plants (pub18-1/+ pub19-1/) produced progeny that numbered significantly more in the parental classes than in the recombinant classes.

B) Arabidopsis chromosomal map showing the distribution of AtPUB18 and AtPUB19 on chromosome 1. Genome image was generated using the Chromosome Map Tool on TAIR (www.arabidopsis.org/jsp/ChromosomeMap/tool).
A

Parental generation: +--- X ---+---

F1 generation: +--- +---

\[ \text{F2 generation:} +--- +--- +--- +--- \]

Parental classes: 19% 46% 32%

Recombinant classes: +--- +--- +--- +---

\[ \text{97%} \]

\[ \text{3%} \]

B

\text{PUB18}

18.7 Mb

\text{PUB19}
Figure 14: Distribution of progeny genotypes from reciprocal back-crosses between double heterozygous plants and wild-type or single homozygous plants.

PCR genotypic screening was performed on individual seedlings from each reciprocated cross. The observed progeny ratios (Observed) were compared to the genotype ratio expected between two alleles with normally independent assortment (Expected-normal) and to that expected when gene linkage is complete (Expected-linked). Chi-square tests were performed comparing Observed genotype distributions to Mendelian expectations when normal or for the hypothetical case of genetic linkage.

A) Genotype distribution of progeny from crosses between pub18-1/+ pub19-1/+ and wild-type. n = 48

Chi-square test of observed distribution for:
“Expected-normal”: $\chi^2 = 43.0$; for degrees of freedom = 3, $P < 0.05$
“Expected-linked”: $\chi^2 = 1.4$; for degrees of freedom = 1, $P > 0.05$

B) Genotype distribution of progeny from crosses between pub18-1/+ pub19-1/+ and pub18-1. n = 44

Chi-square test of observed distribution for:
“Expected-normal”: $\chi^2 = 44.0$; for degrees of freedom = 3, $P < 0.05$
“Expected-linked”: $\chi^2 = 0$; for degrees of freedom = 1, $P > 0.05$

C) Genotype distribution of progeny from crosses between pub18-1/+ pub19-1/+ and pub19-1. n = 39

Chi-square test of observed distribution for:
“Expected-normal”: $\chi^2 = 39.5$; for degrees of freedom = 3, $P < 0.05$
“Expected-linked”: $\chi^2 = 0.23$; for degrees of freedom = 1, $P > 0.05$
A

WT x pub18-1/+ pub19-1/+ progeny

B

pub18-1 x pub18-1/+ pub19-1/+ progeny

C

pub19-1 x pub18-1/+ pub19-1/+ progeny
heterozygous plant \((\text{pub18-1/+ pub19-1/+})\) are askew without the basis of gene linkage came from a second cross performed between a second \(\text{pub18}\) T-DNA line, \(\text{pub18-2}\), and \(\text{pub19-1}\).

To recapitulate the RT-PCR expression analysis showing a positive result when the 5’end of \(\text{AtPUB18}\) was amplified (Figure 5B) and the location of the T-DNA insertion of \(\text{pub18-2}\) at the 3’terminal end of the gene (Figure 4A), there was the possibility that a truncated but still functional protein is being formed in this mutant line. A cross with this second \(\text{pub18-2}\) line yielded progeny closer to the expected Mendelian ratio (Figure 12B). Most notably, \(\text{PUB18/+ PUB19/+}\) progeny as well as \(\text{pub18-2/- pub19-1/-}\) progeny could be generated. Effectively, this second cross became a way to tag and track independent assortment of \(\text{AtPUB18}\) gene without affecting the function of the \(\text{AtPUB18}\) protein. The observed ratio of the progeny from the second self-pollinated tagged plants \((\text{pub18-2/+ pub19-1/+})\) combined with the physical gene distance point to \(\text{AtPUB18}\) and \(\text{AtPUB19}\) not actually being linked genetically.

### 3.7 Further analysis of progeny from self-fertilized double heterozygous plants \((\text{pub18-1/+ pub19-1/+})\) suggests a distortion towards the mutation in \(\text{pub19-1/+}\) progeny

The last observation from the progeny from the second tagged self-pollinated plants \((\text{pub18-2/+ pub19-1/+})\) was that, although the ratio was closer to the expected Mendelian ratio, it was still an altered transmission ratio (Figure 12A). More specifically, the \(\text{PUB18/+ pub19-1/-}\) progeny significantly outnumbered the \(\text{pub18-2/- PUB19/+}\) progeny from the tagged selfed \(\text{pub18-2/+ pub19-1/+}\). Even with the attempt to incorporate the lack of independent gene assortment, progeny from the selfed double heterozygous plants generated in the preliminary cross \((\text{pub18-1/+ pub19-1/+})\) still revealed a similar PUB18/+ pub19-1/- : pub18-1/- PUB19/+ outnumbering. The likely source of this transmission distortion was determined when the progeny from self-pollinated single heterozygous plants \((\text{pub18-1/+ and pub19-1/+})\) were genotyped. The progeny from \(\text{pub18-1/+}\) plants followed the expected 1:2:1 wild-type:heterozygote:homozygote Mendelian segregation ratio (Figure 12D), but the progeny from \(\text{pub19-1/+}\) plants significantly deviated from this ratio and were skewed away from wild-type and towards the homozygous state (Figure 12E). Interestingly, the
distorted segregation ratio of self-pollinated $pub19-1+/+$ heterozygous mutants represents a phenotype that comparing true-breeding non-transgenic sibling (wild-type) plants with true-breeding homozygous plants could not elucidate.

3.8 The generation of a second $pub18+/+$ $pub19+/+$ heterozygous line does not share phenotypes corresponding to the original double heterozygous line

Before continuing the pursuit of any further genotyping of progeny from any additional crosses, including reciprocal crosses between the singly heterozygous plants ($pub18-1+/+$ or $pub19-1+/+$) with wild-type plants to assess whether the transmission of the single $pub19-1$ mutation occurs through the male, female, or both parents, and before attempting extensive investigations into the abortion and collapse seen in the siliques and pollen respectively (ex. quantification, stage of death), it became clear that establishing that these phenotypes seen so far with the original cross ($pub18-1+/+$ $pub19-1+/+$) were not due to additional mutations in the T-DNA mutant background was of paramount importance.

In an attempt to recapitulate the phenotypes seen with the original double heterozygous cross ($pub18-1+/+$ $pub19-1+/+$), an additional double heterozygous cross with another set of T-DNA knock-out lines was pursued ($pub18-3$ and $pub19-3$). Specifically, the phenotypes were a distorted transmission ratio from both the selfed single heterozygous $pub19-1$ plants and the selfed double heterozygous $pub18-1+/+$ $pub19-1+/+$ plants, as well as abortion within the siliques and pollen collapse within the anthers in the latter. Unlike the altered transmission ratio seen in the progeny of selfed $pub19-1+/+$ plants, progeny from $pub19-3+/+$ plants followed the typical 1:2:1 Mendelian segregation ratio (Figure 12F). Similarly, unlike the parental classes dominating over the recombinant classes in the progeny of selfed $pub18-1+/+$ $pub19-1+/+$, progeny from $pub18-3+/+$ $pub19-3+/+$ plants did not show any of these gene-linkage effects, and furthermore, could be classified as falling into the typically expected Mendelian segregation ratio (Figure 12C). Finally, unlike the collapse and abortion seen respectively in the anthers and the siliques in $pub18-1+/+$ $pub19-1+/+$ plants from the original cross, $pub18-3+/+$ $pub19-3+/+$ plants from the additional cross did not show any such gametophytic death in either of these reproductive structures (not shown) and was not seen even when both genes were knocked out ($pub18-3-/+$ $pub19-3-/+$; Figure 10C; Figure 11C).
3.9 Attempts to complement AtPUB18 function with the promoter:DEX system in pub18-1/+ pub19-1/+ plants and to recover double homozygous pub18-1 pub19-1 transgenic plants were unsuccessful

Despite the unsuccessful attempt at recapitulating all the reproduction-related phenotypes seen previously in the first double heterozygous line in an additional double heterozygous line, the question of whether the phenotypes of the former could be directly attributable to either of the pub18-1 or pub19-1 T-DNA mutations remained. Since AtPUB18 and AtPUB19 are expressed in tissue beyond the floral bud, and since the pub18 pub19 double homozygous line might have shown effects beyond the seed reproduction stage, a chemically inducible promoter was used to drive AtPUB18 expression during gametogenesis in an attempt to ensure pub18 pub19 pollen and ovule viability and/or ensure independent assortment of these alleles in the hopes of complementing the genetic-linkage issue found in pub18-1/+ pub19-1/+ progeny and thus generating double homozygous plants (pub18-1 pub19-1). The DEX-driven attempted rescue of potential pub18 pub19 mutants would only require either AtPUB18 or AtPUB19 to be placed under the control of the hormone transcriptional inducer. The further advantage of using conditional complementation was that by withholding chemical inducement, phenotypes associated with the double knock-out could be investigated.

The DEX chemical transcriptional induction system involves a constitutively-expressed chimeric transcription factor, which is composed of the hormone-binding domain (HBD) of the rat glucocorticoid receptor (GR) and the DNA-binding domain of the yeast transcription factor GAL4 and the trans-activating domain of the herpes viral protein VP16, designated GVG. The glucocorticoid receptor is a steroid hormone receptor that also acts as a transcription factor, and the HBD similarly acts as a regulatory domain in the chimeric transcription factor. Thus, in order to activate the chimeric transcription factor to bind the GAL4 upstream activating sequence and subsequently induce the gene of interest transcribed from the promoter containing the activating sequence, dexamethasone (DEX), a strong synthetic glucocorticoid, could be used (above summarized from Aoyama and Chua, 1997). It was presumed that by placing the AtPUB18 cDNA under the control of the DEX inducible promoter and transforming the GVG system into double heterozygous plants (pub18-1/+ pub19-1/+), that spraying
unopened floral buds with DEX and thereby inducing \( \text{AtPUB18} \) expression would help over-ride any genetic linkage-effects at the least and ensure the survival of \( \text{pub18-1 pub19-1} \) ovule and pollen as the ideal. Thus, having viable, independently-assorted gametes, double homozygous \( \text{pub18-1 pub19-1} \) mutants could be rescued with DEX inducible expression past fertilization (Figure 15A).

The \( \text{AtPUB18} \)-containing DEX-GVG system was transformed into \( \text{pub18-1/+ pub19-1/+} \) plants via \textit{Agrobacterium} and the progeny from this dip was expected to be a mixture of genotypes associated with the non-independent assortment of alleles (i.e. singly homozygous for one or the other gene or heterozygous for both genes). From the T2-generation of transgenic seeds collected from the DEX-treated selfed double heterozygous plants, genetic segregation ratios were assessed (Figure 15B). Although full Mendelian proportions were not expected (DEX-induction and therefore “rescue” would not necessarily be 100% effective in every flower), once again the parental classes dominated over the nearly non-existent recombinant classes, indicating that there was no change to the gene linkage-effect seen in the progeny from the original heterozygous plant (Figure 15). Thus, it appeared that the attempt to complement the distorted transmission ratio from selfed double heterozygous (\( \text{pub18-1/+ pub19-1/+} \)) with selective \( \text{AtPUB18} \) expression was unsuccessful.

3.10 A strategy refocused: combining higher order knock-out plants with conditional testing to search for any distinct phenotypic differences

Besides its use as functional complementation, the DEX-GVG-\( \text{AtPUB18} \) strategy was also a proposed means to generate a double knock-out line for use in further studies. Although this system proved to be unfruitful in both aspects, looking back into one of the earliest genotype screens on the progeny from the original double heterozygous plants, seeds that were from a \( \text{pub18-1/+ pub19-1/-} \) plant were found. At the time, amongst the confounding screening results, the appearance of this genotype was deemed to be an anomaly and this plant and its resultant seeds were set aside. In retrospect, given the identification that the alleles in the \( \text{pub18-1/+ pub19-1/+} \) were not independently assorting as expected, we can now understand that this \( \text{pub18-1/+ pub19-1/-} \) plant actually represented a recombinant class that could be naturally generated albeit at a severely decreased frequency. From this selfed heterozygous/homozygous plant,
Figure 15: DEX rescue strategy and results.

A) Strategy illustrating the attempt to rescue non-independent assortment of double heterozygous mutant \((\text{pub}18-1/ + \text{pub}19-1/+\) and generate double homozygous progeny.

The \(PUB18\) gene was cloned into the two-component glucocorticoid-inducible vector pTA7001 a binary GVG transformation vector harbouring the trans-element under the control of the 35S promoter. The DEX chemical transcriptional induction system involves a constitutively-expressed chimeric transcription factor (TF), which is composed of the regulatory hormone-binding domain (HBD) of the rat glucocorticoid receptor (GR) and the DNA-binding domain of the yeast transcription factor GAL4 and the trans-activating domain of the herpes viral protein VP16, designated GVG. To activate the chimeric transcription factor to bind the GAL4 upstream activating sequence and subsequently induce \(PUB18\) transcription from the promoter containing the activating sequence, DEX was used. Double heterozygous plants \((\text{pub}18-1/ + \text{pub}19-1/+\) were transformed and the floral buds of transformants were sprayed with DEX. It was presumed that this strategy would help over-ride any genetic linkage-effects at the least and ensure the survival of \(\text{pub}18-1\ \text{pub}19-1\) ovule and pollen as the ideal. Thus, viable, independently-assorted gametes, double homozygous \(\text{pub}18-1\ \text{pub}19-1\) mutants could be rescued with DEX inducible expression past fertilization.

B) Representative T2-generation transgenic siliques and seeds collected from DEX-treated selfed double heterozygous \((\text{pub}18-1/ + \text{pub}19-1/+\) plant containing the \(AtPUB18\) expression under DEX-inducible control. Bar = 1mm.

C) Genotype distribution of progeny from (B). PCR genotypic screening was performed on individual seedlings isolated from a DEX-treated selfed double heterozygous \((\text{pub}18-1/ + \text{pub}19-1/+\) plant. The observed progeny ratios (Observed) were compared to the genotype ratio expected between two alleles with normally independent assortment (Expected-normal) and to that expected when gene linkage is complete (Expected-linked). \(n = 56\)

Chi-square test of observed distribution for “Expected-normal”: \(\chi^2 = 104.0;\) for degrees of freedom = 8, \(P < 0.05;\) for “Expected-linked”: \(\chi^2 = 8.5;\) for degrees of freedom = 5, \(P > 0.05\)
plants homozygous for both the pub18-1 and pub19-1 mutations could be more readily segregated.

Since the progeny of pub18-3/+ pub19-3/+ screened into ratios that were expected of a typical Mendelian segregation, plants homozygous for the pub18-3 and pub19-3 mutations were easily found. In effect, essentially two double knock-out lines, each derived from independent T-DNA line insertions, were now on hand. Since the caveat was that the pub18-1 pub19-1 double mutant likely has an additional T-DNA insertion, this line could still be used, but to corroborate the pub18-3 pub19-3 double mutant.

3.10.1 No morphological changes associated with the loss of both AtPUB18 and AtPUB19 in optimally grown plants

With these two independent double homozygous lines, observation of general plant development was once again undertaken. Plants grown under optimal conditions on soil however, again showed no phenotypic differences between each double mutant line to wild-type plants. This ranged from non-flowering 3-week-old leaves (Figure 8A), 4-week-old rosettes (Figure 8E), to 6-week-old flowering plants (Figure 9D). As well, silique dissection revealed no abortion seen to the extent observed in pub18-1/+ pub19-1/+/+ plants (Figure 10B). And likewise, Alexander’s staining of the anthers revealed no pollen collapse (Figure11B). Thus, reinforcing once more that the reproduction-related outcomes seen in pub18-1/+ pub19-1/+/+ were likely unrelated to either AtPUB18 or AtPUB19 function.

3.10.2 Identifying growth conditions ideal for eliciting differences in double knock-out pub18 pub19 lines

Having selected T-DNA insertion lines that best represent knock-outs in AtPUB18 and AtPUB19 expression and having generated double knock-out plants to address for any functional redundancy between closely related AtPUB-ARM proteins, a remaining possibility to reveal phenotypes for homozygous mutant plants was to use specific environmental conditions that differ from the ideal growth conditions. To narrow the large scope of experiments that could be used in elucidating gene function, we looked at data generated from microarray transcript profiling projects, accessed through the Expression Browser tool available at the Bio-Array Resources for Plant Functional
Upon examination of the data sets accumulated to date on the Bio-Array Resource (BAR) database, notable up-regulation for \textit{AtPUB18} and \textit{AtPUB19} was seen for select hormonal and abiotic stress treatments.

A query of the hormone series found in AtGenExpress data set (Goda et al., 2008) yielded ABA-related increases in \textit{AtPUB18} and \textit{AtPUB19} mRNA levels. Likely because \textit{AtPUB19} transcript levels appear to be much more elevated than those for \textit{AtPUB18}, expression of the latter could not be as readily associated to the same co-expression with ABA treatment (Figure 3C), however, absolute levels from the ABA time course in wild-type seedlings illustrate the up-regulation of expression in both \textit{AtPUB18} and \textit{AtPUB19} (Figure 16A, 16B).

Intriguingly, when the UND-containing \textit{AtPUB-ARMs} were queried in the wild-type seedling time courses of the stress series of the AtGenExpress data set (Kilian et al., 2007), \textit{AtPUB19} and \textit{AtPUB18} showed the best correlative expression (Figure 3A). Selective increases in transcript levels were seen for salt (Figure 17B, 17D), osmotic (Figure 18B, 18D), and (to a lesser extent) cold and drought stresses for \textit{AtPUB19} within 24 hours of treatment in both shoot and root tissues. Similarly, \textit{AtPUB18}'s expression profile, although mRNA levels were not as strongly increased, followed the same pattern of increase under these same four abiotic stress conditions (Figure 3A). While \textit{AtPUB18} mRNA up-regulation was clearly seen in relative values in salt (Figure 6C) and osmotic (Figure 18C) treatments, absolute values revealed that expression increases are most evident in root tissues (Figure 17A; Figure 18A).

### 3.10.3 Loss of both \textit{AtPUB18} and \textit{AtPUB19} expression showed decreased sensitivity to both ABA and salt treatment during germination

Based on the observations that ABA and abiotic stress treatments increased \textit{AtPUB18} and \textit{AtPUB19} transcript levels, one hypothesis was that both \textit{AtPUB18} and \textit{AtPUB19} might function during defensive mechanisms against abiotic stresses. Thus the intent was to establish that without the stress-inducible \textit{AtPUB18} and \textit{AtPUB19} proteins, knock-out seeds would be either more or less sensitive to these conditions. As such, the effects of losing \textit{AtPUB18} and \textit{AtPUB19} expression were tested during the response of \textit{Arabidopsis} to ABA and salt treatments. First, germination assays were
Figure 16: Expression patterns for AtPUB18 and AtPUB19 in response to ABA hormone treatment.

The schematic representations of expression from the Abiotic Stress data set from the At-Tiling Array Express (At-TAX; Zeller et al., 2009) were generated with the Arabidopsis eFP Browser at BAR (www.bar.utoronto.ca).

Expression changes in 10-day-old whole seedling tissues of plants after the addition of 100μM of ABA to the growth media.

Absolute values:

A) AtPUB18 expression with and without treatment

B) AtPUB19 expression with and without treatment

Relative values:

C) AtPUB18 expression (with treatment relative to without)

D) AtPUB19 expression (with treatment relative to without)
### Absolute

**A**

**Control**

![Absolute Control 0 Hour](image)

![Absolute Control 1 Hour](image)

![Absolute Control 12 Hours](image)

**ABA (100 μM)**

![Absolute ABA 0 Hour](image)

![Absolute ABA 1 Hour](image)

![Absolute ABA 12 Hours](image)

### Relative

**C**

**ABA (100 μM)**

![Relative ABA 0 Hour](image)

![Relative ABA 1 Hour](image)

![Relative ABA 12 Hours](image)

**D**

**ABA (100 μM)**

![Relative ABA 0 Hour](image)

![Relative ABA 1 Hour](image)

![Relative ABA 12 Hours](image)
**Figure 17:** Expression patterns for AtPUB18 and AtPUB19 in response to salt stress treatment.

The schematic representations of expression from the Abiotic Stress Series data set (Kilian *et al.*, 2007) were generated with the *Arabidopsis* eFP Browser at BAR ([www.bar.utoronto.ca](http://www.bar.utoronto.ca)).

Expression changes in 18-day-old shoot and root tissues of plants after the addition of 150mM of NaCl to the growth media.

Absolute values:

**A)** *AtPUB18* expression with and without treatment

**B)** *AtPUB19* expression with and without treatment

Relative values:

**C)** *AtPUB18* expression (with treatment relative to without)

**D)** *AtPUB19* expression (with treatment relative to without)
**Absolute**

**A**  
*AtPUB18*

Control  
Root  
0 Hour  0.5 Hour  1 Hour  3 Hours  6 Hours  12 Hours  24 Hours  
Salt (150mM NaCl)  
Root  
0 Hour  0.5 Hour  1 Hour  3 Hours  6 Hours  12 Hours  24 Hours  

**B**  
*AtPUB19*

Control  
Root  
0 Hour  0.5 Hour  1 Hour  3 Hours  6 Hours  12 Hours  24 Hours  
Salt (150mM NaCl)  
Root  
0 Hour  0.5 Hour  1 Hour  3 Hours  6 Hours  12 Hours  24 Hours  

**Relative**

**C**  
*AtPUB18*

Salt (150mM NaCl)  
Root  
0 Hour  0.5 Hour  1 Hour  3 Hours  6 Hours  12 Hours  24 Hours  

**D**  
*AtPUB19*

Salt (150mM NaCl)  
Root  
0 Hour  0.5 Hour  1 Hour  3 Hours  6 Hours  12 Hours  24 Hours
Figure 18: Expression patterns for AtPUB18 and AtPUB19 in response to osmotic stress treatment.

The schematic representations of expression from the Abiotic Stress Series data set (Kilian et al., 2007) were generated with the Arabidopsis eFP Browser at BAR (www.bar.utoronto.ca).

Expression changes in 18-day-old shoot and root tissues of plants after the addition of 300mM of mannitol to the growth media.

Absolute values:

A) AtPUB18 expression with and without treatment

B) AtPUB19 expression with and without treatment

Relative values:

C) AtPUB18 expression (with treatment relative to without)

D) AtPUB19 expression (with treatment relative to without)
Absolute

A

AtPUB18

[Diagrams showing plant growth under control and osmotic (300 mM Mannitol) conditions for shoot and root over 0.5, 1, 3, 6, 12, and 24 hours]

B

AtPUB19

[Diagrams showing plant growth under control and osmotic (300 mM Mannitol) conditions for shoot and root over 0.5, 1, 3, 6, 12, and 24 hours]

Relative

C

AtPUB18

[Diagrams showing plant growth under osmotic (300 mM Mannitol) conditions for shoot and root over 0.5, 1, 3, 6, 12, and 24 hours]

D

AtPUB19

[Diagrams showing plant growth under osmotic (300 mM Mannitol) conditions for shoot and root over 0.5, 1, 3, 6, 12, and 24 hours]
done with seeds from both double knock-out lines, *pub18-1 pub19-1*, *pub18-3 pub19-3*, and both single knock-out lines for the latter, *pub18-3*, *pub19-3*, in response to the presence of increasing concentrations of ABA (1-10 μM) and NaCl (100-150mM). In both assays, control treatment-free germination of mutant seeds on the first day showed significant variation from the germination of wild-type seeds (Figure 19A, Figure 20A). This indicated that assessment of stress response during germination on the first day with treatment would not be a reliable indicator of seed germination changes associated with either ABA or salt treatment since variability in germination after day one could be attributable to factors outside of these treatments. Germination essentially reached 100% by the second day for all lines tested when no treatment was examined, and in fact, no significant change in treatment-free germination was seen when wild-type and mutant seeds were compared, indicating that the set of seeds were equally viable and suitable for conditional germination testing to eke out any stress-related effects only (Figure 19A).

Endogenously, ABA is necessary for dormancy induction, however low levels of exogenous ABA is sufficient to suppress seed germination and serves as a convenient quantitative assay for sensitivity of the ABA response (Finkelstein *et al.*, 2002). In general, upon exposure to exogenous ABA, seeds from *pub18-1 pub19-1* and *pub18-3 pub19-3* double knock-out lines showed greater germination efficiency when compared to wild-type and single knock-out lines (Figure 19B, 19C). The decreased sensitivity to ABA during germination of double knock-out lines was most notable in the highest concentration of ABA (10μM), with significant differences detected from the third to seventh day of germination, and by day 10, germination of all seed lines were equalized (Figure 19C). With an intermediate concentration of ABA (1μM), the same significant increase in germination is seen in *pub18-1 pub19-1* and *pub18-3 pub19-3* from the second to third day of germination before all lines germinated equally by day 4 (Figure 19B). Interestingly, the germination of the *pub18-3* and *pub19-3* single lines also showed significant differences from wild-type germination with the intermediate ABA concentration at day 2 and at day 3 for the latter. This decreased germination in *pub19-3* was replicated but it would be interesting to see if this same decline in
**Figure 19: Decreased sensitivity of pub18 pub19 transgenic lines to ABA treatment.**

Germination ratio of wild-type, *pub18-1 pub19-1, pub18-3 pub19-3, pub18-3,* and *pub19-3* lines in the absence or presence of ABA was determined.

**A)** 0μM ABA control (100% methanol)  

**B)** 1μM ABA  

**C)** 10μM ABA  

Counts were taken daily over the course of one week and on the tenth day of germination. Radicle emergence was scored as seed germination. The values are means ± standard error. Three replicates were performed, with each replicate comprising 48 seeds. The asterisk (*) denotes statistical significance from wild-type (*P* < 0.05) as determined by a two-tailed and unpaired Student’s *t*-test.
A

Wildtype  pub18-1  pub19-1  pub18-3  pub19-3  pub18-3  pub19-3

Germination

Day

control

B

Wildtype  pub18-1  pub19-1  pub18-3  pub19-3  pub18-3  pub19-3

Germination

Day

1 μM ABA

C

Wildtype  pub18-1  pub19-1  pub18-3  pub19-3  pub18-3  pub19-3

Germination

Day

10 μM ABA
Figure 20: Decreased sensitivity of pub18 pub19 transgenic lines to salt stress.

Germination ratio of wild-type, pub18-1 pub19-1, pub18-3 pub19-3, pub18-3, and pub19-3 lines in the absence or presence of NaCl was determined.

A) 0mM NaCl

B) 100mM NaCl

C) 125mM NaCl

D) 150mM NaCl

Counts were taken daily over the course of four days. Radicle emergence was scored as seed germination. The values are means ± standard error. Three replicates were performed, with each replicate comprising 48 seeds. The asterisk (*) denotes statistical significance from wild-type ($P < 0.05$) as determined by a two-tailed and unpaired Student’s $t$-test.
A) Wildtype
  pub18-1
  pub19-1
  pub18-3
  pub19-3
  pub18-3
  pub19-3

control

B) Wildtype
  pub18-1
  pub19-1
  pub18-3
  pub19-3
  pub18-3
  pub19-3

100mM NaCl

C) Wildtype
  pub18-1
  pub19-1
  pub18-3
  pub19-3
  pub18-3
  pub19-3

125mM NaCl

D) Wildtype
  pub18-1
  pub19-1
  pub18-3
  pub19-3
  pub18-3
  pub19-3

150mM NaCl
germination relative to wild-type could be similarly ascertained in seeds from the other pub19-1 T-DNA line. The increase in germination of pub18-1 pub19-1 and pub18-3 pub19-3 double knock-out lines and not in pub18-3 or pub19-3 single knock-out lines is in line with redundancy existing between these two proteins. These results indicated that there was decreased sensitivity to the germination-inhibiting effects of ABA when AtPUB18 and AtPUB19 functions were both nullified.

In Arabidopsis, high concentrations of salts inhibit germination (Quesada et al., 2000; Zhu, 2000; Xiong et al., 2002). Overall, while the mildest salt stress concentration (100mM NaCl) only slightly affected the germination efficiency of most lines (Figure 20B), germination of the both double knock-out lines was less reduced with high salt stress concentrations (125-150mM NaCl), an increase in germination efficiency that was not seen in the single knock-out lines (Figure 20C, 20D). Like ABA, the highest concentration of salt yielded the most distinct germination changes in seeds from both the pub18-1 pub19-1 and pub18-3 pub19-3 double knock-out lines from the second to third day of germination before all lines germinated equally at day 4 (Figure 20D). And like ABA, the intermediate concentrations of salt treatment resulted in perplexing germination efficiencies. While the mildest salt concentration alone (Figure 20B) may be too low to solely define the change in germination of seeds from pub18-3 upon salt stress, the decreased germination seen in seeds from pub19-3 is quite dramatic at day 2 and remains even when a higher salt concentration was used (Figure 20C). As with seed germination with ABA, it will be of interest to see if germination of seeds from pub19-1 would also demonstrate such a comparable drop in germination efficiency during salt stress. Nonetheless, when considering seeds from both double knock-out lines, germination was significantly higher than wild-type at all salt concentrations tested. And like ABA, the increase in germination of the double knock-out lines and not in single knock-out lines is in line with there being redundancy between these two proteins. These results indicated that the loss of both AtPUB18 and AtPUB19 lead to decreased sensitivity to salt during germination.
3.10.4 No change in response to ABA and abiotic stresses with the loss of both AtPUB18 and AtPUB19 expression during root growth

Having observed a decrease in sensitivity to both ABA and salt stress during seed germination, the next step was to determine whether these results could be reconciled with the previously examined profiles found from microarray data that focused exclusively on gene expression of the root and shoot tissue of transferred seedlings. Thus, as a second part of the assessment of Arabidopsis responses to abiotic stresses, root growth assays were performed on double knock-out lines (pub18-1 pub19-1, pub18-3 pub19-3) and both single knock-out lines for the latter (pub18-3, pub19-3), where 3-day-old seedlings were transferred to media supplemented with a range of ABA (0.1-10μM) and NaCl (50-100mM) concentrations. The intent was to follow-up on the seed germination results and establish whether the loss of both AtPUB18 and AtPUB19 expression also be able to overcome the inhibitory effects of these same ABA and salt treatments during seedling root growth.

With the addition of exogenous ABA to the growth media, although the mild stress stimulation of low ABA concentrations can enhance root growth, shoot growth and root elongation is inhibited with severe stress simulation of higher ABA concentrations (Watts et al., 1981; Hooker and Thorpe, 1998) through the effects of this phytohormone on reducing cell division and elongation (Finkelstein et al., 2002). Relative root elongation of transferred seedlings after 3, 5, and 7 more days of growth was recorded to determine seedling response to the hormone treatments. Unlike that seen with germination, there were no remarkable phenotypic differences in relative root growth between the double knock-out lines from wild-type or the single knock-out lines with increasing ABA concentrations (Figure 21A).

With NaCl exposure, Arabidopsis seedlings show general growth inhibition, including reduced root length, reduced leaf size, and chlorosis of leaves (Shi et al., 2003; Gao et al., 2003; Brini et al., 2007). As a convenient indicator of seedling growth, relative root elongation of transferred seedlings was recorded to determine seedling response to the salt treatments. Double knock-out, wild-type, and single knock-out seedlings all exhibited similar root growth sensitivity upon additional salt stress conditions (Figure 21B).
Figure 21: Lack of effects with ABA treatment, salt stress, and osmotic stress on pub18 pub19 transgenic seedling root elongation.

Relative root length growth of wild-type, pub18-1 pub19-1, and pub18-3 pub19-3 lines in the absence or presence of various microarray-directed abiotic stresses was determined. Three-day old seedlings were subjected to root growth assays with and without ABA, NaCl, and mannitol and the growth of roots was monitored.

A) ABA treatment (0.1-10μM)

B) salt stress (50-100mM NaCl)

C) osmotic stress (150-300mM mannitol)

Root measurements were taken on the third day of additional vertical growth. Relative root elongation was calculated as average length of additional growth with treatment over average length of additional growth without treatment (ie. MS only or control). The values are means ± standard error. Three replicates were performed, each replicate comprising 6 seedlings. No statistical significance from wild-type ($P < 0.05$) was seen as determined by two-tailed and unpaired Student’s $t$-tests.
As an additional means to test seedling growth sensitivity upon exposure to abiotic stress, a root length assay with varying osmotic treatments (150-300mM mannitol) was performed. Similar to mild ABA stress, mild osmotic stress inhibits leaf growth while allowing roots to grow and elongate (Hsiao and Xu, 2000), but like salt stress, higher osmotic stress levels leads to reduced elongation during seedling development (Rosado et al., 2006). Similar to root growth seen with ABA and salt exposure, double knock-out mutants did not display any detectable changes in root growth in comparison with wild-type seedlings in response to osmotic stress (Figure 21C).

In contrast with what was observed in seed germination, pub18-1 pub19-1 and pub18-3 pub19-3 seedlings did not differ in sensitivities to selected abiotic stresses from wild-type seedlings during early root growth. This juxtaposition between seed germination and root elongation results has been seen before (Samuel et al., 2008).

### 3.10.5 Testing additional abiotic stress responses in the pub18 pub19 mutants

To see if other sources of abiotic stresses might be able to elicit increased or decreased sensitivities, oxidative and heat stresses during root growth were also investigated. In previous studies, AtPUB22 and AtPUB23 were linked to regulating drought, and to a lesser extent salinity, stress responses (Cho et al., 2008). The interaction seen between AtPUB22 and AtPUB23 with a 19S regulatory particle subunit, RPN12a (Cho et al., 2008), linked the oxidative and heat stress response testing of such proteasomal subunit mutants to a possible novel PUB function, where PUBs are capable of modifying plant stress tolerance between ubiquitin-dependent 26SP protein degradation involved in salt, drought, and heat stress responses and ubiquitin-independent 20SP protein degradation involved in oxidative stress responses (Yee and Goring, 2009).

Specifically, researchers studying the proteasomal mutants used specific compounds that could simulate oxidative and heat stresses to uncover phenotypes (Kurepa et al., 2008; Wang et al., 2009).

As a redox-active compound, methyl violagen (MV) enters the plant cell where, due to photosynthetic activity, the divalent cation takes on electrons and is readily reduced to form the stable MV free radical that subsequently reacts with oxygen to regenerate MV
and produce a superoxide radical capable of inducing oxidative stress (Slade, 1966). Consistent with the accumulation of ROS upon salt and drought stresses (Borsani et al., 2001), low levels of ROS could act as effective secondary messengers to elicit modifying stress responses, whereas high levels would bring about damaging oxidative stress (Zhu, 2002). As an amino acid analog, L-canavanine (CAN) is incorporated into proteins instead of arginine leading to protein misfolding (Rosenthal et al., 1989) capable of eliciting a cellular response similar to that caused by heat stress (Peng et al., 2001; Kurepa et al., 2003).

Like the root growth assays for ABA treatment, salt sensitivity, and osmotolerance, these same assays testing for changes in susceptibilities to methyl viologen (Figure 22A) and L-canavanine (Figure 22B) did not show any differences in the effects these compounds have on the growth of roots from double knock-out seedlings. Given the lack of significant AtPUB18 and AtPUB19 gene expression induction associated with oxidative and heat stress in the publicly available microarray data sets (Figure 6), this lack of root growth phenotype is not unexpected.

3.10.6 Testing the effects of ABA biosynthesis inhibition and light conditions on pub18 pub19 mutant root growth

Having attempted to elicit morphological changes in the double knock-out lines by exposing them to singular abiotic stress tests, the next approach was to begin combining the double knock-out lines with additional mutations and with other stresses.

Upon closer examination of the information available from publicly released projects in the data sets accumulated to date on the Bio-Array Resource (BAR) database, increased levels of AtPUB19 transcripts were also seen in ABA-related mutants. In particular, this up-regulation of AtPUB19 was seen in aba2-2, an ABA auxotroph with defects in ABA biosynthesis (McCourt Lab, http://bar.utoronto.ca/affydb/cgi-bin/affy_db_proj_viewer.cgi?view=general&proj=26 and http://bar.utoronto.ca/affydb/cgi-bin/affy_db_proj_viewer.cgi?view=general&proj=30). In both projects, normally grown aba2-2 seedlings were subjected to ABA treatment along with a transcriptional inhibitor (actinomycin D) for the latter or a protein synthesis inhibitor (cyclohexamide) for the former. Without interference from the presence of physiological ABA, the induction of
Figure 22: No effects on pub18 pub19 transgenic seedling root elongation with oxidative stress, heat stress, and ABA inhibition.

Relative root length growth of wild-type, *pub18-1 pub19-1*, and *pub18-3 pub19-3* lines in the presence of various abiotic stresses showing no previous *AtPUB8* and *AtPUB19* expression induction and in with the addition of ABA inhibition was determined. Three-day old seedlings were subjected to root growth assays with and without methyl viologen, L-canavanine, and fluridone and the growth of roots was monitored.

A) oxidative stress (0.2-1.5μM methyl viologen (MV))

B) heat stress (1-8μM L-canavanine (CAN))

C) ABA inhibition (1-100μM fluridone (FLU))

Root measurements were taken on the third day of additional vertical growth. Relative root elongation was calculated as average length of additional growth with treatment over average length of additional growth without treatment (ie. MS only or control). The values are means ± standard error. Three replicates were performed, each replicate comprising 6 seedlings. No statistical significance from wild-type (*P < 0.05*) was seen as determined by two-tailed and unpaired Student’s *t*-tests.
A. 

oxidative stress

Relative root length growth

Day 3

MS + 0.2uM MV | + 0.6uM MV | + 1.5uM MV

B. 

heat stress

Relative root length growth

Day 3

MS +1uM CAN | + 4uM CAN | + 8uM CAN

C. 

ABA biosynthesis inhibition

Relative root length growth

Day 3

0 (MetOH) | + 1uM FLU | + 10uM FLU | + 100uM FLU
AtPUB19 was seen in aba2-2 mutants in regardless of transcription or transcription inhibition, but only when ABA was exogenously applied. This suggests that AtPUB19 up-regulation can be a directly ABA-dependent response and can also be independent of transcriptional rates.

As well, a recently published paper revealed the importance light conditions have in determining whether mutants in another PUB protein (AtPUB44/SAUL1) display a leaf senescence phenotype (Raab et al., 2009). In this situation it was low light that triggered premature leaf senescence upon the loss of the PUB gene. However, in remaining with the inclination of identifying additional conditions that promote AtPUB19 expression, plants adjusting to high-light conditions also up-regulate AtPUB19 all while accumulating ABA (Galvez-Valdivieso et al., 2009). Thus changing light levels in general might be an important criterion to consider when dealing with conditional testing of ABA-responsive genes, which AtPUB19 and, and to a lesser extent, AtPUB18 are.

Given that my project timeline no longer allowed for the genetic generation of a triple mutant between pub18 pub19 mutants and ABA-deficient mutants, a chemical approach to impose ABA deficiency was used as an alternative. Initially discovered as an herbicide (Waldrep and Tailor, 1976), fluridone is a carotenogenesis inhibitor (Bartels and Watson, 1978), and as carotenoids are precursory to the biogenesis of ABA in plants, ABA synthesis is also detrimentally affected (Gamble and Mullet, 1986). Thus, fluridone was used to lower endogenous ABA levels in the double knock-out lines to see if this combinatory loss of AtPUB18 and AtPUB19 expression with ABA auxotrophy might be successful in generating morphological changes. Upon fluridone treatment, root elongation of plants is inhibited (Hooker and Thorpe, 1998; Spollen et al., 2000) and photobleaching subsequently occurs as the lack of carotenoid accumulation allows for the photooxidation and destruction of chlorophyll (Popova and Riddle, 1996). After 3 days of growth on fluridone-supplemented media, inhibited root growth and the beginnings of photobleaching were seen in the double negative lines but with no difference with respect to the wild-type response (Figure 22C).

In conjunction with the findings that Arabidopsis leaves adjusting to high-light accumulates ABA and that both ABA and high-light treatments up-regulate AtPUB19
(Galvez-Valdivieso et al., 2009), the light-dependence of double *pub18* *pub19* knock-out lines to exhibit a root growth phenotype during salt stress was investigated. In our experimental set-up, in addition to testing high-light conditions from the onset (Figure 23D), changes in light levels were also tested. Thus, regular-light-acclimatized seedlings were subjected to high-light conditions while under salt stress (Figure 23B) and vice versa (Figure 23C). As previously seen with salt-stress alone (Figure 21B), seedling growth diminished with increasing salt concentration, however, no differences between the double knock-out lines were seen in comparison to wild-type regardless of the change in light conditions used (Figure 23).

### 3.11 *pub18* *pub19* mutant leaf responses to senescence, given AtPUB19's strong expression at this stage

Besides using publicly-available microarray projects to establish conditional root assay and germination testing parameters, other transcript profiling data were assessed to look for other directions from which to elucidate gene function. One such approach is the observation of strong AtPUB19 expression in senescing leaves (Figure 6D). In general, mutations in AtPUB18 and AtPUB19 appear to reduce leaf chlorophyll content, with no apparent further reduction with *pub18* and *pub19* combination mutations (Figure 24D). To induce a senescence partially mimicking nature, leaves were detached and light-deprived before total chlorophyll content was measured (Buchanan-Wollaston et al., 2005; Pruzinska et al., 2005). With increasing time under dark treatment, leaves increasingly lose green pigment due to the degradation of chlorophyll (Figure 24). Visually, no obvious differences could be seen between single and double mutants and wild-type dark-induced senescence (Figure 24A, 24B, 24C), while chlorophyll loss assessment also did not reveal any clear senescence response (Figure 24).
Figure 23: No difference in salt-stress acclimation is seen in pub18 pub19 transgenic seedling root elongation when considering light-dependency.

Relative root length growth of wild-type, pub18-1 pub19-1, and pub18-3 pub19-3 lines in the absence or presence of salt stress was determined. Three-day old seedlings initially grown under regular- or high-light conditions were subjected to root growth assays with and without NaCl and continued under the same light conditions or changed to the alternate light condition after transfer. The growth of roots was monitored.

A) regular-light conditions

B) regular-light-acclimatized seedlings transferred to high-light conditions

C) high-light-acclimatized seedlings transferred to regular-light conditions

D) high-light conditions

Root measurements were taken on the third day of additional vertical growth. Relative root elongation was calculated as average length of additional growth with treatment over average length of additional growth without treatment (ie. MS only or control). The values are means ± standard error. Three replicates were performed, each replicate comprising 6 seedlings. No statistical significance from wild-type ($P < 0.05$) was seen as determined by two-tailed and unpaired Student’s $t$-tests.
A. Regular-to-regular light

B. Regular-to-high light

C. High-to-regular light

D. High-to-high light

Wildtype **pub18-1** **pub19-1** **pub18-3** **pub19-3**
Figure 24: No significant differences in dark-induced leaf senescence is seen in pub18 pub19 transgenic plants.

Loss of chlorophyll from leaves detached from wild-type, pub18-1 pub19-1, pub18-3 pub19-3, pub18-3, and pub19-3 lines was observed upon induction of senescence by darkness.

A) Visual appearance of leaves at the start of the experiments

B) Visual appearance of senescing leaves after 3 days of darkness

C) Visual appearance of senesced leaves after 7 days of darkness

D) Chlorophyll content for (A-C)

Total chlorophyll was extracted and measurements were taken in leaves at the start and 3 and 7 days after senescence was induced and normalized against the fresh weight of each leaf. The values are means ± standard error. Three replicates were performed, each replicate comprised a whole leaf.
CHAPTER 4: DISCUSSION

4 Discussion

Ubiquitin-mediated proteolysis is an integral part of diverse cellular functions, and of the three enzymes involved in linking ubiquitin to protein targets, the E3 ubiquitin ligases are of particular interest as they confer substrate specificity during this process. The E3 ubiquitin ligases can be categorized based on mechanism of action and on the presence of specific domains such as the RING, HECT, F-box, and U-box domains. In plants, the U-box family has undergone a large gene expansion that may be attributable to biological processes unique to the plant life cycle. There are 64 predicted plant U-box proteins in Arabidopsis, and the biological roles of many of these have yet to be determined. Research on PUB genes from several different plants has started to elucidate a range of functions for this family, from self-incompatibility and hormone responses to defence and abiotic stress responses. Expression profiling has also been used as a starting point to elucidate PUB function, and has uncovered a strong connection of PUB genes to various stress responses.

Through this study, attempts to elucidate the precise role of AtPUB18 and AtPUB19 during plant growth, development, and acclimation were made. From a combination of sequence similarity and of co-expression at the expression level under certain conditions, it is likely that AtPUB18 and AtPUB19 have partially redundant functions. From condition-based expression studies, AtPUB18 and AtPUB19 are likely involved in abiotic stress-related responses. My research has demonstrated that these two E3 ubiquitin ligases are capable of sharing similar functions, consistent with redundancy seen in other similarity-based sub-groupings of other AtPUB genes. Furthermore, I have shown that without both AtPUB18 and AtPUB19, there is decreased sensitivity to seed germination under certain germination-detrimental conditions; the purpose of this negative regulation has yet to be determined. Thus, this work has begun to shed insight on the roles of AtPUB18 and AtPUB19 during mediation of abiotic stress acclimation in Arabidopsis and my findings are discussed below.
4.1 A role for PUB E3 ubiquitin ligases during gametophytic development?

To list the biological processes in which ubiquitin-26S proteasome system is involved would be the equivalent of listing every process in which gene transcription and translation plays a role. With the volume of UPS research being generated, it is evident that the regulated degradation of proteins plays a huge role in governing plant growth, development, and acclimation, just as the regulated synthesis of proteins does (Smalle and Vierstra, 2004; Mazzucotelli et al., 2006; Stone and Callis, 2007; Vierstra, 2009). The fundamental importance of ubiquitin-mediated degradation as a regulatory mechanism in itself is not hard to ascertain, but what drives researchers is the discovery of the precise details behind the many orchestrated interaction networks governed by 26S-proteasome directed ubiquitination during plant growth, development, and acclimation. The most outstanding question following from this research is establishing upon which protein targets and under what conditions and circumstances (and thus during which signal transduction pathways) the numerous E3 ubiquitin ligases exert their substrate-specific ubiquitination activity.

The formation of the 26S proteasome complex would logically play a role during selective protein degradation, and recently, the RP lid subunit RPN5 has been implicated in 26SP stability, and the inactivation of one Arabidopsis isoform showed distinct impairment during gametogenesis, one of many defects that could partially be explained by altered 26SP integrity (Book et al., 2009). Given the importance of proper 26S proteasome assembly for gametophyte development, it seemed reasonable to connect the early discovery of a proportion of collapsed non-viable pollen grains in the anthers and a proportion of ovule abortion in the siliques from self-crossed pub18-1/+ pub19-1/+ heterozygous plants to AtPUB18 and AtPUB19 involvement in gametogenesis (Figure 10D; Figure 11D). What gave even more credence to this reasoning was that a plant’s ability to reproduce is diminished upon environmental stress, and this accelerated infertility has been correlated with degenerative aborting of ovule and pollen development during times of water stress as induced by salt and other osmotica (Sun et al., 2004), two types of abiotic stresses upon which both AtPUB18 and AtPUB19 are both up-regulated upon exposure (Figure 3A).
With these observations, the implication was that the loss of a single copy of \textit{AtPUB18} and one copy of \textit{AtPUB19} might lead to ovule abortion and pollen collapse even when there was no external abiotic stress afflicting the plant. Results from the genotypic analysis of the various mutant allele combinations from a selfed \textit{pub18-1/+ pub19-1/+} heterozygote are consistent with the prospect that gametes lacking both genes are most susceptible to premature arrested development. Inviability of \textit{pub18-1 pub19-1} ovules and pollen grains would easily explain the failure to identify screened seedlings homozygous for \textit{pub18-1} and \textit{pub19-1} or homozygous for one mutation and heterozygous for another (\textit{pub18-1/+ pub19-1/-} or \textit{pub18-1/- pub19-1/+} allele combinations) (Figure 12A). Thus, this led to initial thoughts that \textit{AtPUB18} and \textit{AtPUB19} function might be activated upon abiotic stress signalling to ubiquitinate and target downstream components that would normally allow/promote gametophytic misdevelopment for destruction, in an attempt to minimize gametophyte loss during reproduction in environmentally detrimental conditions. However, these results could not be verified with the other \textit{pub18 pub19} mutant alleles.

4.2 A role for PUB E3 ubiquitin ligases during meiotic recombination?

As a way to foster genetic diversity, eukaryotic sexual reproduction relies on meiotic recombination to bring together homologous chromosomes to allow redistribution of their allelic contents as part of the reductional division of diploid precursors into haploid gametes that are genetically different from one another. In a self-fertilizing plant like \textit{Arabidopsis}, the nature of the wild-type background ensures generation of homogenous progeny matching the parental genotype, but all the genetic-exchanging events associated with homologous recombination prior to gamete formation still occur during meiosis. And despite the initial appearance of genetic disarray that could be attributed to such random reorganization of allelic combinations, the transmission of genes, and any mutations they carry, from parent to offspring does typically follow Mendelian inheritance. Thus, with an understanding of independent assortment and chromosomal crossover, one can generally follow and predict the ratio of each possible genetic combination in the progeny from the self-fertilization outcome of a double mutant dihybrid cross.
A full examination of the genetic segregation of the various mutant allele combinations in the progeny from the selfed pub18-1/+ pub19-1/+ heterozygote still demonstrated an aberrant percentage, however, even when the expected ratio is adjusted for pub18 pub19 gametophytic non-viability (Figure 12A). The discrepancy between numbers for both single homozygous progeny, which would otherwise be projected to be equal, could be attributed to an underlying condition of one of the parental mutant lines. The propensity towards the pub19-1 mutation is not understood, but the increased occurrence of the pub19-1 mutation in progeny from selfed pub19-1/+ plants, likely reflects why the pub18-1 mutation (pub18-1/- PUB19-1/+ pub19-1/) segregated at three-fifths the frequency of the pub19-1 mutations (PUB18-1/+ pub19-1/-) in progeny from selfed pub18-1/+ pub19-1/+ plants (Figure 12A, 12E).

What was harder to explain was how the numbers of the progeny genotypes in themselves were so suggestive of genetic linkage between AtPUB18 and AtPUB19. With the progeny falling into parental classes heavily outnumbering those in recombinant classes both in pub18-1/+ pub19-1/+ self-crosses (Figure 13A) and back-crosses with wild-type and single homozygotes (Figure 14), the evidence was suggestive that AtPUB18 and AtPUB19 were autosomally linked because of their shared residence on the same chromosome. On the other hand, the considerably large distance between the two genes on the same chromosome (Figure 13B) precluded AtPUB18 and AtPUB19 as a linkage group. With these considerations, an alternate explanation was that AtPUB18 and AtPUB19 are involved in the independent assortment of alleles during meiosis. Consistent with this, crossing the same pub19-1 mutation with a weaker allelic pub18-2 mutation recovered F2 progeny that more closely followed the segregation of the various mutant allele combinations of independently assorting alleles, with any slight distortion perhaps being attributable to penetrance of the pub18-2 mutation (Figure 7B) or the bias towards the pub19-1 mutation (Figure 12B). More specifically the non-independent assortment of AtPUB18 and AtPUB19 alleles in the double heterozygote during meiosis is characteristic of a lack of inter-chromosomal recombination that would otherwise occur with chromosomal crossovers.

The process of meiotic recombination itself involves a complex but well-orchestrated series of chromosomal events, from the juxtapositional pairing of homologous
chromosomes, to the recombinatorial exchange of allelic counterparts between homologous pairs, ending with the random but accurate segregation of these chromosomes away from each other at the first meiotic division. Essential to the actual physical interaction (chiasmata) and reciprocal trading of genetic material between homologous regions is the crossover formation; mediation of which involves several proteins during the DNA double-strand break (DSB) formation, followed by end processing into the single-stranded DNA involved in strand invasion into a chromatid for DSB repair in Arabidopsis (Mezard et al., 2007). In the occasion of aberrant recombination and/or missegregation, the circulation of such severe genetic defects would logically be unfavourable to plant propagation. Preventing the continued development of such abnormal gametes and removing them from availability during reproduction would be desirable. Concomitant with the pollen collapse and ovule abortion seen in pub18-1/+ pub19-1/+ plants (Figure 10D; Figure 11D), gametophytic non-viability and decreased fertility caused by male and female meiotic defects are associated with disruptions in crossover events, as qualified by chiasmata and bivalent formation (Grelon et al., 2001; Chen et al., 2005; Stacey et al., 2006).

When considering the connection between up-regulation of AtPUB18 and AtPUB19 during abiotic stress and meiosis, the correlation between such induced somatic response and programmed gametophytic development could be the shared role of DNA damage repair. The DSB formation of crossovers during meiotic recombination is intrinsically a form of DNA damage that needs to be properly repaired to maintain genomic integrity prior to meiotic division. At the same time, although UV-B is the abiotic stress most usually associated with the induction of DNA lesions, DNA damage, such as nicking, and the accumulation of double-strand-break-inducing ROS, has been detected in salt-stressed ovules (Sun et al., 2004; Sun et al., 2005; Tuteja, 2007). Correspondingly, when a biochemically active pea plant DNA helicase, whose superfamily contains members that have regulatory roles in uncoiled-DNA-requiring events such as recombination and repair (Tuteja, 2007), or rice DNA topoisomerases, whose Arabidopsis homologs (AtSPO11) have been implicated in meiotic recombination (Grelon et al., 2001; Stacey et al., 2006), were transformed into and over-expressed in tobacco or Arabidopsis respectively, increased tolerance to abiotic stresses, specifically
salt and osmotic treatments, was observed (PDH45, Sanan-Mishra et al., 2005; OsTOP6A3, OsTOP6B, Jain et al., 2006; OsTOP6A1, Jain et al., 2008). Supporting the association between stress and meiosis, AtATM, the Arabidopsis homolog of a human protein (Ataxia Telangiectasia Mutated) involved with DNA repair activities (Kastan and Lim, 2000), is important in both exogenously-induced and endogenously-programmed DNA damage responses (Garcia et al., 2003).

With this information, the implication would be that, with the loss of a single copy of AtPUB18 and one copy of AtPUB19 during gametogenesis, any DNA-repair-related activity might be attenuated enough during crossover formation that meiotic recombination would be defective and resultant aberrant gametes would be programmed to abort. The gene-linkage-like results from the genotypic analysis of the various mutant allele combinations from a selfed pub18-1/+ pub19-1/+ heterozygote are consistent with the prospect that gametes with recombinant combinations were rendered non-functional due to crossover defects, while the gametes which assorted with non-recombinant parental combinations were unaffected. Thus, another possibility was that AtPUB18 and AtPUB19 function to ubiquitinate and target for destruction downstream negative regulators of DNA repair activities, and their increased expression upon abiotic stresses is an adjustment to counter DNA damage incurred from such negative impacts of environmental stress.

4.3 Finding additional independent T-DNA mutations is not uncommon

Upon failure to conditionally complement the non-independent assortment of pub18-1/+ pub19-1/+ (Figure 15) and regenerate ovule abortion (Figure 10B, 10C), pollen collapse (Figure 11B, 11C), and the altered transmission ratio (Figure 12C) in double homozygous plants with two other T-DNA independent mutations (pub18-3 pub19-3) or even with the same T-DNA mutations (pub18-1 pub19-1), it quickly became apparent that neither of the outlined scenarios above was reflective of AtPUB18 and AtPUB19 function during plant growth, development, and acclimation. With closer analysis, the reliance of the normally-constitutive and nearly-ubiquitous 35S promoter to drive the expression of the DEX-responsive chimeric transcription factor was eventually pinpointed as one weakness of this complementation strategy as it was discovered that
the activity of the promoter is attenuated in plant gametophytic tissues (Bent, 2000),
where factors preventing gamete death (i.e. ovule abortion, pollen collapse) and/or
promoting proper independent gene assortment prior to meiosis would presumably be
at play. The failure of the DEX-conditional attempt at rescuing gametophytic
development-related phenotypes in the original \textit{pub18-1/+ pub19-1/+} heterozygote is
more likely due to the complementation with a gene (\textit{AtPUB18}) that is unrelated to their
causative mutation(s). What is more likely valid is that these aberrant gametophytic
outcomes are due to the existence of another T-DNA insertion, and thus disruption of
another gene in the original T-DNA double heterozygous line (\textit{pub18-1/+ pub19-1/+})
and of the initial cross lines. Given the near allelic independent assortment in the F2
progeny of the original \textit{pub19-1} mutation crossed with an alternative \textit{pub18-2} mutation
(\textit{pub18-2/+ pub19-1/+}), specifically this additional T-DNA insertion mutation can likely
be traced to the \textit{pub18-1} T-DNA line.

It appears that this phenomenon of a severely decreased progeny distributed across
recombinant classes during the construction of double mutant T-DNA lines has been
encountered before (Samuel et al., 2008; Curtis et al., 2009). When an \textit{ark9} and \textit{abi3-6}
homozygous T-DNA insertions lines were crossed, as for \textit{pub18-1} and \textit{pub19-1}, aborted
seeds, only parental genotypes and next to no recombinant genotypes were seen in the
resulting generation (Samuel et al., 2009). While the non-Mendelian progeny
misdistribution towards parental classes is not as severe, another lab found that their
cross between lines with homozygous T-DNA insertions for \textit{AtREV3} and \textit{AtPOLH}
yielded an anomalous progeny ratio with recombinant classes being generated at a
decreased frequency (Curtis et al., 2009). From their findings they were able to predict
that a T-DNA-induced chromosomal rearrangement had occurred, where translocation
resulted from homologous recombination between independent T-DNA insertion
insertions. For this to have occurred, they identified a possible additional T-DNA
insertion in one of their crossing lines, with spreads of this mutant in heterozygous form
showing the cruciform four-chromosome structure during meiosis that could result form
a reciprocal translocation.

The generation of T-DNA mutagenized \textit{Arabidopsis thaliana} insertion lines involves the
\textit{Agrobacterium tumefaciens}-mediated transformation with a T-DNA vector construct
containing an antibiotic-resistant marker gene for quick screening of tagged mutants. Their use in reverse-genetics methodology has greatly facilitated the efficiency of plant functional genomics research (SAIL, McElver et al., 2001; SALK, Alonso et al., 2003; GABI-Kat, Rosso et al., 2003). Advantages to this type of insertional mutagenesis are the essentially random independent incorporation events across the entire genome and the near saturation of the gene space due to large scale. Furthermore, each mutation can be precisely mapped to genome locations by sequencing the T-DNA-border-adjacent plant DNA fragments flanking insertion sites, through which each line can be identified and pooled into databases of sequence-indexed collections, further simplifying BLAST-supported searches. A disadvantage to this type of randomized mutation is that more than one insert can be incorporated, which means more than one gene could theoretically be disrupted. The average number of T-DNA insertion per line is ~1.5 in the SALK population (Alonso et al., 2003), 1.3-1.8 in the GABI-Kat collection (Rosso et al., 2003), and assumed to be around 1.5-2.0 in the SAIL lines from Syngenta (McElver et al., 2001), which do not account for tandem insertions (more than one copy of T-DNA insert at the same site) or second-site insertions in closely linked sites that would behave as a single genetic locus.

It is not unusual for alternate T-DNA insertion mutations to exist and to influence outcomes that are capable of setting researchers off-course from properly and effectively studying the function of their gene or genes of interest. For example, in contrast to work subsequently done on RPN1a, the original study showed embryonic lethality in a T-DNA line despite this mutation not generating a fully nullified gene (Brukhin et al., 2005; Wang et al., 2009). The inability to recapitulate embryonic lethality in other independent alleles that were full knock-outs as well as in the same rpn1a-3 line (Wang et al., 2009) could be explained by the presence of an supplementary background mutation in a gene essential for embryogenesis, a characteristic that caused a different and unrelated phenotype to the initial gene of interest. Not every T-DNA insertion results in gene disruption, but nor are extra supplementary mutations limited to only one additional locus. Raab et al. (2009) found that one of their SALK T-DNA insertion lines in the SAUL1/PUB44 gene contained three additional T-DNA
insertions located in the three genes adjacent to their gene of interest that all resulted in other gene deletions.

To avoid re-evaluation and dismissal of any role with which a protein has been ascribed through the use of T-DNA insertion lines, there are many ways to rule out that a phenotype discovered in a single T-DNA insertion line is related to alternate mutations in other genes. One way is to disprove the presence of any additional T-DNA insertions in the same T-DNA line; this could be done with Southern blotting or TAIL-PCR experiments. A second way is to minimize the chance existence of any further T-DNA insertions through backcrossing with wild-type for several generations to clean up the genetic background; with each subsequent backcross, only half of the ensuing propagated population would have the offending additional mutation in the background, allowing its frequency to halve with each backcross. Alternatively, another way one can prove that the initial phenotype discovery was not a coincidence of any other mutations; this can involve finding the same phenotype in another allelic but independent T-DNA insertion mutant or using backcrossing and co-segregation analyses. And last but not least, is to prove the loss-of-function is correlated with the T-DNA insertion mutation through complementing the phenotype by reintroducing the loss-of-function mutation with an active protein. In the event that accompanying T-DNA insertions are found and localized to other genes, work on other knock-out T-DNA mutants matching these genes could be done to prove that these latter gene disruptions are not related to the initial phenotype of interest.

From the above examples, Raab et al. (2009) used alternate knock-out T-DNA lines in each of the genes with additional T-DNA insertions to prove that the insertion in the gene of interest and not the deletions of adjacent genes was pertinent to the senescence seen in this line. To follow up, they also used a second T-DNA line free of further insertions as an independent allele and backcrossed this line with wild-type to show the light-dependency segregated with the T-DNA mutation and complemented the phenotype with a constitutively expressing SAUL1/PUB44 gene. To counter the claims made by Brukhin et al. (2005) that homozygous rpn1a seed are embryo lethal and any seed that do set are revertants, Wang et al. (2009) pointed to contradictory results where another group showed that the homozygous rpn1a-2 mutation is capable of
surviving past the embryonic stage (Huang et al., 2006), as also seen for a second homozygous rpn1a-3 mutation. To further reinforce the re-evaluation of RPN1a function, they used two other rpn1a T-DNA lines that represented null alleles of the RPN1a gene. For my work, additional T-DNA lines that were independent alleles to the original were used in an attempt to recreate the original phenotypes and a conditional rescue attempt was launched to see if the restoration of AtPUB18 gene to a wild-type status might complement these phenotypes. With the lack of success of either method to support the notion that gametophytic non-viability and disrupted independent allelic assortment is correlated to AtPUB18 and AtPUB19 function, the presence of additional T-DNA insertions in the genome is the likely cause of these phenotypes. To follow up that the pub18-1 T-DNA line has additional insertions, the crossing of this line into an additional pub19 line (for example pub19-2) has been done. Any appearance of aborted ovules, collapsed pollen, and gene-linkage-like propagation in the progeny of such a double heterozygous line would lend credence to focus on this line to search for and identify any additional T-DNA inserts and to correlate these phenotypes with the causative mutation.

4.4 Circumventing a lack of altered growth responses by addressing redundancy

Crosses between pub18 and pub19 mutants were originally done as a means to address functional redundancy since their single loss-of-function mutants, like the majority of mutants used in reverse genetics approaches, lacked any visibly distinguishable morphological changes. AtPUB18 and AtPUB19 were chosen because of their shared homology (Figure 4B), their mutual clustering from sequence similarity (Figure 5), and their shared gene expression (Figure 2). Not surprisingly, even with the loss of both genes, no direct phenotypic alterations were exhibited in standard growth conditions (Figures 8-9), which follows the notion that various mutations need to be tested in a wide range of environments before exhibiting conditional phenotypes (Bouche and Bouchez, 2001). Although AtPUB19 is a higher expressor than AtPUB18, their co-expression was seen with select abiotic stresses (Figure 3A). Given how, of all the AtPUB genes, AtPUB19 had the highest up-regulation upon salt and ABA
treatments, it was surprising that two knock-out lines in both genes did not show any changes in susceptibility or resistance to salt or ABA in root length assays (Figure 21).

With no change in seedling growth associated with the loss of both AtPUB18 and AtPUB19, it is possible that neither of these two E3 ubiquitin ligases have a function in the response to abiotic responses. With germination assays, however, response to both ABA and salt inclusion in the growth media was altered, most notably at higher concentrations (Figure 19C; Figure 20C, 20D). Compared to wild-type and the single knock-out lines tested, seeds from both pub18-1 pub19-1 and pub18-3 pub19-3 lines exhibited significantly decreased sensitivity to ABA and salt treatment. This decrease in ABA and salt sensitivity in loss-of-function mutants supports a role for both AtPUB18 and AtPUB19 in abiotic acclimation and suggests a negative regulatory role for AtPUB18 and AtPUB19 in germination during ABA and salt responses.

Paradoxically, differences with respect to wild-type were seen in seed germination with the double knock-out lines during ABA and salt treatment (Figures 19-20) despite differences in expression levels between AtPUB18 and AtPUB19 being seen in dry and imbibed seeds (Figure 6E). And even more surprisingly, no differences with respect to wild-type were seen in root elongation with the double knock-out lines upon ABA and salt exposure (Figure 21A, 21B, 23) despite both genes being distinctly expressed in root tissues (Figure 6B, 6G). It is not clear why such an unpredictability could exist, however, the specificity of the decreased ABA and salt sensitivity phenotype seen in the double knock-out lines to seed germination and not root growth in seedlings has been seen before in another ABA-responsive PUB, AtPUB9 (Samuel et al., 2008).

An alternate possibility explaining why changes were seen in germination rates and not root growth in the presence of abiotic stress treatments is additional functional redundancy during specific phases of plant development. In the scenario of germinating seeds overcoming inhibition to ABA and salt, AtPUB18 and AtPUB19 have overlapping functions during ABA and salt responses, though one cannot rule out that higher-order PUB knock-out combinations might display stronger instances of insensitivity. For example, there may be an increase in either the scale to exhibit a larger extent of insensitivity, or in the range to become more perceivable at lower concentrations or be
still perceivable at higher concentrations. This suggests that higher order knock-outs are perhaps necessary before changes in their abiotic responses during seedling root growth are noticeable. Recently, *pub22 pub23* root growth patterns and germination were examined for their response to salt and ABA treatment, respectively, but did not differ in appearance from wild-type despite the *AtPUB22* and *AtPUB23* genes being up-regulated in seedlings and seedling roots under salt conditions (Figure 2C; Cho *et al.*, 2008). When another group examined a similar double mutant coupled with another mutation in a closely related gene, *AtPUB24*, the *pub22 pub23 pub24* triple mutant exhibited an enhanced oxidative burst, an immune response triggered by pathogen invasion that, upon being discovered in the higher-order triple mutant, could be subtly ascertained in the lower-order *pub22 pub23*, *pub22 pub24*, and *pub23 pub24* double mutants (Trujillo *et al.*, 2008).

Just like it would be interesting to see if this *pub22 pub23 pub24* triple knock-out mutant would display germination and root growth abberations during salt stress testing, it would be interesting to see if an additional *pub* mutation in conjunction with *pub18 pub19* mutations would display root elongation changes during salt treatment. The provision of higher-order mutants can be justified upon recalling the seed germination assays during ABA and salt treatments (Figure 19-20). While the increased insensitivity of seeds from double knock-out lines germinating on ABA and salt was readily seen, seeds from the single knock-out lines germinating on ABA and salt were more inconsistent. The difference in seed germination from the *pub18-3* and *pub19-1* lines was more easily ascertained after discovering the more significant seed germination increase in the *pub18-1 pub19-1* and *pub18-3 pub19-3* lines. As such, the reasoning behind the disparate contrast between single knock-out lines and double knock-out lines without corroborating seed germination results from the untested *pub18-1* and *pub19-1* lines remains unknown; however, the necessity of higher-order mutants may be the element to address before changes can be elucidated in the abiotic stress testing root elongation assays.

Based on overall sequence similarity, AtPUB18 and AtPUB19 are clustered together and away from the 39 other AtPUB-ARM sequences analyzed (Mudgil *et al.*, 2004). A similar clustering of AtPUB18 and AtPUB19 occurs when similarity is based on ARM
repeat domain sequences of the 17 UND-containing AtPUB-ARM genes (Figure 5D). By respective gene comparison with regards to U-box and UND domain sequences, AtPUB8 (45% similarity whole gene comparison or 76% similarity comparison from U-box onward) and AtPUB16 (82% similarity)/AtPUB17 (79% similarity) are successive structurally similar candidates (Figure 5A, 5B). From microarray analyses, AtPUB45 (70% similarity) demonstrates the nearest co-expression during select abiotic stresses and while the PCC is low, a visual comparison between the relative to control results of AtPUB2 (74% similarity) suggests these might be the most probable partially functionally redundant candidates (Figure 3A, 3C).

By mutually considering both sequence homology and overlapping co-expression patterns there is no clear consensus AtPUB gene knock-out triple combination to be additionally pursued unilaterally. Instead, perhaps what needs to be considered is a more experiment by experiment approach to suggest what other AtPUB may be involved. Identifying such a basis for any additional knock-out mutation to be coupled with pub18 and pub19 mutations may be the key to ascertaining further protein redundancies. For example, during cold responses, the homologous gene of interest would be AtPUB45 and perhaps might even require a quadruple mutation with AtPUB2 (Figure 3A). And for osmotic and salt stress and ABA treatment responses, AtPUB2 gene selection as the additional knock-out would be more appropriate (Figure 3A, 3C).

In the latter scenario, the inclusion of the loss of AtPUB2 along with AtPUB18 and AtPUB19 during germination and root elongation assessments upon ABA and salt treatments (Figures 19-21) would be a remarkable choice since the expression of AtPUB2 is not detected in dry and imbibed seeds (Figure 25Av) and at first glance is not as pronounced in root tissues (Figure 25Aii, 25Avii) when compared to that of AtPUB18 and AtPUB19 (Figure 6). But from the Abiotic Stress data set from At-Tiling Array Express, AtPUB2 expression is present in whole seedling tissues (Figure 25B) and from the Abiotic Stress Series data set, AtPUB2 expression is detectable in root tissues upon salt treatment (Figure 25C), making the additional selection of this PUB gene a valid candidate. Further considerations would be observing beyond the relative control output of microarray results (Figure 3) and weighing those results against what is seen in absolute levels (Figure 16-18) since the former can mask interesting patterns in the
Figure 25: Expression patterns of AtPUB2 (At5g67340) during various stages and tissues of plant development and in response to ABA hormone and salt stress treatments.

All generated with the Arabidopsis eFP Browser at BAR (www.bar.utoronto.ca).

A) As per Figure 6. The schematic representations of absolute expression from the Development Series data set. (i) Stage 12 (top) and stage 15 (bottom) flowers showing expression levels for sepals, petals, stamens, and carpels (Schmid et al., 2005); (ii) 7-day-old (left) and 17-day-old (right) seedlings showing expression levels for cotyledons, hypocotyl, and roots of the former and vegetative rosette and roots of the latter (Schmid et al., 2005); (iii) Siliques (top) with stage 4 seeds (bottom) (Schmid et al., 2005); (iv) Cauline (left) and senescent (right) leaves (Schmid et al., 2005); (v) Dry (top) and 24-hr-imbibed (bottom) seed (Nambara lab http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=183); (vi) Central zone (top), rib meristem (middle), and peripheral zone (bottom) of the shoot apical meristem (SAM) of ap1-1 cal1-1 (a double mutant that converts floral meristems to shoot meristems) (Yadav et al., 2009); (vii) Root cell type expression from the quiescent center, and the stele, endodermis, cortex, epidermal artrichoblasts, epidermis (II, III), and lateral root cap (I, II) (cell types from center out to periphery) of stage I, II, III roots (stages increasing from tip upwards) (Birnbaum et al., 2003; Nawy et al., 2005); (viii) Leaf cross sections showing expression in mesophyll and guard cells after control (top) and ABA treatment (bottom) (Yang et al., 2008).

B) As per Figure 16. The schematic representations of expression from the Abiotic Stress data set from the At-Tiling Array Express (At-TAX; Zeller et al., 2009). Absolute expression changes in 10-day-old whole seedling tissues of plants after the addition of 100µM of ABA to the growth media.

C) As per Figure 17. The schematic representations of expression from the Abiotic Stress Series data set (Kilian et al., 2007). Absolute expression changes in 18-day-old shoot and root tissues of plants after the addition of 150mM of NaCl to the growth media.
latter and absolute levels can provide a context for relative fold changes. Thus, diligent analysis of the information provided by the microarray results need to be taken into account.

Overall, considerable deliberations into possible structurally and functionally redundant PUB E3 ligase candidates need to be undertaken to determine the most relevant higher-order mutants to be pursued.

The question then arises about whether or not it makes sense to have so many functionally redundant or partially functionally redundant genes governing the same processes. Given their sessile nature, plants have evolved modifications to allow changes in their physiology to counter changes in their environmental conditions in lieu of physically changing their location to escape any profoundly altering abiotic or biotic situations. Often counteracting potentially detrimental growth conditions requires plants to redirect their resources away from normal growth, development, and reproduction to consolidate energy into survival and adaptive responses. Thus it follows logic that, being so environmentally sensitive, plants use redundancy to remain capable of launching protective biotic and abiotic responses, thus precluding random mutations in one gene from entirely preventing their ability to sense and react appropriately to stress conditions.

4.5 Over-expression instead of loss-of-function knock-outs as a source of conditional changes

While loss-of-function mutants are preferably suited to study gene function by correlating visible associated phenotypes directly to functional necessity, over-expression studies are also compatible with identifying possible gene function through functional sufficiency. In abiotic stress testing, ideally, the loss-of-function mutation could be attributed to change in susceptibility to abiotic treatment and gain-of-function mutations and/or over-expression of the gene exhibit an opposing responsiveness. The causal connection between loss-of-function and gain-of-function is not always simultaneous, and in the aforementioned pub22 pub23 study, while loss of both genes did not yield any changes in root growth during their response to salt stress, over-expression of either AtPUB22 or AtPUB23 caused increased sensitivity (Cho et al.,
Considering that pub18 pub19 mutants have shown no changes in root growth upon so many types of abiotic stress – salinity, osmotic, oxidative, heat, ABA (Figure 21-22), it would be interesting to see if transgenic plants constitutively expressing either AtPUB18 and/or AtPUB19 might confer phenotypic changes upon these same treatments.

For germination, increased insensitivity to ABA and salt treatments was seen in seeds combining both the pub18 and pub19 mutations (Figure 19-20). But it still remains to be further substantiated that it is definitively the loss of both the AtPUB18 and AtPUB19 that is directly causing these observed phenotypes. Complementation of phenotypes could be accomplished by re-introducing either normal or constitutive AtPUB18 or AtPUB19 expression into the double null lines. As for further support for the germative role of AtPUB18 or AtPUB19 during abiotic stress conditions, a function-modified (ex. loss of U-box function through conserved amino acid modification) gene copy could be re-introduced similar to what has previously been done in various PUBs (BnARC1-P323A, BnARC1-Δ236-347, Stone et al., 2003; OsSPL11-V290R, OsSPL11-Δ314-316, Zeng et al., 2004; CaPUB1-T61A, Cho et al., 2006; AtPUB17-V322I, Yang et al., 2006; AtPUB22-V24I, AtPUB23-B29I, Cho et al., 2008; AtPUB22-W40A, AtPUB22-C13A, Trujillo et al., 2008) This approach would also provide insights into structure-function associations.

4.6 The emerging relevance of light conditions and ABA

The condition-dependent nature of most knock-out mutations generated for reverse genetics approaches means that a wide variety of environmental conditions might need to be tested before any conditional phenotypes can be revealed. While some mutations might need as little as one alteration from standard growth conditions, other loss-of-function mutations might need a specific multi-combinatorial approach to varying growth parameters before eliciting morphological changes in comparison to wild-type response. In this work, the search for similar gene expression patterns was used to focus on select environmental factors before analyzing mutants. While germination assays only required an addition of salt or ABA into the growth media to demonstrate pub18 pub19 seeds have increased insensitivity to these stresses (Figure 19-20), root elongation...
assays with the same mutants with an equivalent addition of salt or ABA alone did not so readily demonstrate growth changes (Figure 21A, 21B). An attempt to combine ABA-insensitivity with the double mutant lines also showed no differences with respect to wild-type (Figure 22C), however, root growth testing here occurred in otherwise normal growth conditions. Thus it will be interesting to see if combining ABA-deficiency imposed by fluridone treatment with abiotic stress (example: salinity) to see if two simultaneous conditions that had elicited AtPUB18 and AtPUB19 up-regulation in microarray data (salt stress and endogenous ABA-deficient mutants) might reveal informative phenotypes.

In the course of preliminary vertical root growth assays with salt, one consideration that arose was whether light level adjustments might be required to elicit changes in root growth sensitivity or tolerance. Since then, an early senescence phenotype seen in saul1/pub44 mutants has been shown to be entirely dependent on photon flux density (PFD), with completely normal development at high PFD and leaf yellowing at low PFD (Raab et al., 2009). Combining high-light stress with salinity stress did not yield any root growth patterning differences between double null lines and wild-type (Figure 23), however, other light factors may prove to be more auspicious, including decreased light intensity levels or altered light cycle regime from long-day to short-day conditions. Considering the influence of light or of other environmental factors, including such extra adjustments into the seedling growth assays may be necessary to reveal the conditional phenotypes that may prove to be most informative of gene function.

4.7 Conclusion

In the work presented, AtPUB18 and AtPUB19 appear to be redundantly involved in abiotic stress responses, specifically during seed germination. With the loss of functional AtPUB18 and AtPUB19, Arabidopsis plant growth and development appeared normal under standard growth conditions (Figures 8-11). Under abiotic stress growth conditions, aberrations in seedling and root growth were not seen with the deletion of these genes (Figures 21-22). Interestingly, the absence of both AtPUB18 and AtPUB19 in seeds, however, results in increased germination capabilities in comparison to wild-type seeds during germination-detrimental abiotic stresses, at least with salt stress and
with ABA treatment (Figures 19-20). It has been previously established that the phytohormone ABA inhibits seed germination and that various abiotic stresses are capable of increasing ABA biosynthesis and accumulation (Finkelstein et al., 2002). From research presented in this thesis, it has been shown that AtPUB18 and AtPUB19 are up-regulated upon exposure to ABA (Figure 2, 3C, 16) and salt (Figure 2, 3A, 17), amongst other abiotic stresses (Figure 2, 3A, 18). Cumulatively, this suggests that AtPUB18 and AtPUB19 redundantly function to inhibit germination during abiotic stress conditions and thus, appear to act as possible negative regulators of abiotic stress acclimation (Figure 26).

Similar to AtPUB22, AtPUB23, and AtPUB24, it is perplexing how the loss of both AtPUB18 and AtPUB19 results in increased resistance to normally inhibitory effects of ABA and salt while the expression of these genes is increased in response to these same stresses (Cho et al., 2008; Yee and Goring, 2009). AtPUB22, AtPUB23, AtPUB24 are suggested to target subunits of 19S proteasome regulatory particles, which couple with the 20S core particle to form the 26SP, and through this modulation result in a balance of maintaining priority between the mediation of high temperature stress resistance through ubiquitin-dependent 26SP degradation or the mediation of oxidative stress resistance through ubiquitin-independent 20SP degradation (Kurepa and Smalle, 2008b; Yee and Goring, 2009). Although the target substrate(s) of AtPUB18 and AtPUB19 are not yet known, evidence for a similar 20SP/26SP-based modification to alter stress acclimation between temperature/oxidative stress has not been seen (Figure 22). This, however, does not mean that PUB18/19 could not have a similar acclimative role during the balancing of other abiotic responses.

Considering that it is essential that plants have multiple mechanisms in place to ensure optimal growth, development and acclimation to a variety of environments, the prevention of seed germination in growth-inhibiting environmental conditions is thus justifiable. As E3 ubiquitin ligases, regulatory function performed by AtPUB18 and AtPUB19 could be taken from a more stress-responsive perspective (Figure 26A) or from a more hormone-based perspective (Figure 26B), both of which would effect the germination outcome of seeds. By preventing germination in suboptimal conditions,
Figure 26: Conceptual representation of possible AtPUB18 and AtPUB19 involvement during abiotic stress responsive signalling in plants or during germination responses in seeds.

With the understanding that AtPUB18 and AtPUB19 are U-box E3 ubiquitin ligases, they would govern the ubiquitination of a yet unknown target substrate. Without having yet identified a substrate it would be unknown whether this ubiquitination is the polyubiquitination associated with 26S proteasome degradation of the substrate or the monoubiquitination associated with protein function modification (localization, activity, interaction) of the substrate. Based on the germination assays under salt or ABA conditions, whether it be negative regulation of positive regulators or positive regulation of negative regulators, part of the function of AtPUB18 and AtPUB19 appears to be the overall negative regulation of germination of seeds during stress responses. From the microarray data, it appears abiotic stresses like salt and abiotic-stress related ABA up-regulate AtPUB18 and AtPUB19 expression. Arrowed lines represent stimulatory positive effects. Blunted lines represent repressive negative effects.

A) Focusing more on the abiotic stress-responsive events, abiotic stresses (such as salinity) are sensed, resulting in changes in ABA accumulation and response (through increased biosynthesis / decreased catalysis or signalling). Alterations in ABA results in the intermediate signalling for changes in the expression of abiotic stress-related genes (including AtPUB18 and AtPUB19) and activation of various downstream stress responses (such as growth inhibition, cellular detoxification and repair, and damage control). Alternatively, such stress responsive gene regulation can be directly mediated by the abiotic stress perception in an ABA-independent manner. Supporting the possibility of ABA-independent stress induction is the up-regulation of AtPUB18 and AtPUB19 during abiotic stress that appears to occur faster than ABA biosynthesis (E. Nambara personal communication). The collective responses are integrated together to balance homeostasis of the overall plant health to optimize stress tolerance and survival.

B) Focusing more on the germination-responsive events (B), abiotic stresses (such as salinity) are sensed, resulting in changes in AtPUB18 and AtPUB19 accumulation and
activity. Based on the results of this research, AtPUB18 and AtPUB19 appear to act as negative regulators of seed germination during ABA and salt treatment, thus may be positive regulators of ABA biosynthesis or signalling or may be negative regulators of ABA catalysis. Because ABA is capable of influencing AtPUB18 and AtPUB19 expression), a positive feedback loop may be amplifying the effects of ABA during germination. As an inducer of seed dormancy, the presence of ABA prevents the germination of seeds. Seed germination is also largely controlled by other phytohormones, most notably GAs. While ABA initiates seed dormancy, GA breaks seed dormancy by promoting germination. Thus by targeting the biosynthesis, catalysis, and/or signalling of other germination-relevant phytohormones, AtPUB18 and AtPUB19 may be influencing the germination outcome of seeds in response to environmental conditions.
A

stress perception intermediate signalling stress responses

salt → ABA biosynthesis / catalysis or signalling

AtPUB18

26SP? monoUb?

AtPUB19 ? → germination

? → germination

+ growth inhibition
+ detoxification
+ damage control
+ repair
+ etc.

salt → ABA-independent upstream signalling stress responsive gene up-regulation

stress tolerance

acclimatized homeostasis to balance overall plant survival

integration and balancing of responses

B

stress perception intermediate signalling hormonal responses germination outcome

salt → PUB18 PUB19 ? → ABA → germination

↑ ′ve feedback loop

Biosynthesis (+'ve) or Signalling (+'ve) or Catalysis (-'ve) or Integration of other germination-relevant hormones ex. ABA counter hormone during germination: Germination-promoting GA vs Dormancy-promoting ABA
these possible examples of ubiquitin-related regulation would actually ensure overall optimal plant growth, development, and acclimation collectively.

It is currently unknown as to what, if any, plant phenotypes or seed germination profiles over-expression of AtPUB18 or AtPUB19 would yield; whether they would yield hypersensitivity to abiotic stresses by demonstrating decreased germination in the presence of ABA or salt or not demonstrate any growth alterations or defects in relation to increased susceptibility to abiotic stresses. For now, my research on two plant E3 ubiquitin ligases with overlapping functions has shown how complex the nature of combining specific protein function redundancy with specialized abiotic stress responses during environmental acclimation can be in *Arabidopsis*.

### 4.8 Additional Future Directions

While this work has begun the process of correlating AtPUB18 and AtPUB19 function to abiotic stress responses in *Arabidopsis*, a considerable number of questions, as suggested and discussed previously and partially reiterated and expanded here, remain to be resolved. For future research prospects, the overall goal for AtPUB18 and AtPUB19 will be to further define the roles these E3 ubiquitin ligases have in the seed germination pathway during salt and ABA treatments, as well as to characterize further details surrounding their function during general abiotic stress responses. Additionally, the role of AtPUB18 and AtPUB19 can be examined within a larger context of emerging themes of PUB function, like programmed cell death or homeostasis disruption, to compare with a more generalized mechanism of action found with other plant U-box E3 ubiquitin ligase members.

With the loss of both AtPUB18 and AtPUB19, seed germination has decreased sensitivity to the inhibitory effects of salt and ABA treatments. Even with this observation that AtPUB18 and AtPUB19 may function to counter germination during detrimental environmental conditions, the increased germination phenotype of double knock-out seed is not yet fully informative and requires other supporting work to substantiate any claims of negative regulatory AtPUB18 and AtPUB19 function during abiotic stresses. Having generated plants with two double knock-out combinations of
mutations in *AtPUB18* and *AtPUB19* and a DEX-inducible construct driving *AtPUB18* expression, the initial groundwork for future expansive and exploratory work has been laid.

First, while further germination assays could be performed to determine what other abiotic stresses might demonstrate decreased germination sensitivity with the loss of *AtPUB18* and *AtPUB19*, to ultimately prove that the loss-of-function mutations of these specific genes is causative to increased germination insensitivity to abiotic stresses, complementation of the phenotype by reintroducing an active protein needs to be completed. For the first half of this objective, the most obvious other abiotic stress to test would specifically be osmotic stress to illustrate that the water-balance equilibrium and not the ionic component of the salt treatment is responsible for disrupting germination. Given a similar germination effect with the abiotic-stress-related ABA hormone, it is doubtful that ionic disequilibrium caused by increased sodium and chloride ions is the primary cause, however testing with other salts (examples: Li⁺ as an analog to Na⁺, K⁺, Cs⁺) should not be entirely overlooked. Also, oxidative and high-temperature related treatments during germination would round out the abiotic stress testing and provide the contrast in abiotic stresses not related to *AtPUB18* and *AtPUB19* up-regulation (Figure 3B). For the second half, either the *AtPUB18* or *AtPUB19* active protein could be reintroduced under its own natural promoter, a constitutive promoter, or a conditional promoter. To this end, the DEX construct conditionally driving *AtPUB18* expression has already been introduced into both double knock-out mutant lines, and from here stably transformed lines can be screened and induced to demonstrate any regain of a more typical germination effect during one or all abiotic stress tests.

Second, as previously discussed, while germination assays on seeds over-expressing either PUB protein might demonstrate further decreased germination during inhibitory abiotic stress treatments, vertical root elongation assays with these same over-expressor seedlings might be the key to generating seedling growth phenotypes to further illustrate *AtPUB18* and *AtPUB19* function during abiotic stress defence mechanisms. Showing increased sensitivity to abiotic stresses during germination with over-expressor seeds would demonstrate *AtPUB18*/*AtPUB19* sufficiency in negatively
controlling germination during abiotic stress responses and thus would be a nice corollary to AtPUB18/AtPUB19 necessity already seen with combined loss-of-function mutations during germination under abiotic stress (Figures 19-20). Meanwhile, any morphological seedling changes associated with AtPUB18 and/or AtPUB19 over-expression would be a welcome clue to further understanding of what role these PUBs play during abiotic signalling. Ideally, germination and root elongation assays would produce results that would be complimentary to one another and to the assessment of gene function. Currently, although perhaps not as ideal as the constitutive 35S promoter to provide over-expression, the DEX-inducible AtPUB18 expression introduced into wild-type plants has already been performed. With transformant seeds ready to be screened for their AtPUB18-induction by DEX and upon establishment of stably transformed lines, executing germination assays on these conditionally-over-expressing lines could be sufficient to demonstrate any increased sensitivity of protein over-availability during seed germination and any effects in early seedling growth.

Third, as briefly touched upon in the conclusions, with AtPUB18 and AtPUB19 involvement during seed germination during abiotic stress responses, involvement of these same E3 ubiquitin ligases during seed germination during hormonal responses may also be relevant. And with an association with seed germination, the contribution of seed dormancy should also be considered when studying the function of these proteins. In particular, the germination results of double knock-out lines and of the single knock-out lines in the assay controlling for seed germination capacity is of interest. In both controls for the ABA and salt treatment germination assays (Figure 19A, 20A), significant variation in germination was seen for all tested lines after the first day of germination before settling into the equalized germination capacity of the second day. This germination variability points to the possible involvement of inconsistent seed dormancy. Since the germination assays used in this research performed stratification to break dormancy and ensure non-delayed germination, germination assays without the use of such seed imbibition under cold and dark conditions should be explored. Furthermore, to properly assess degree of dormancy of seeds, considerations into after-ripening effects during seed storage durations would need to be included. Although performed in the deeply dormant Cape Verde Island (Cvi) accession of Arabidopsis
thaliana, the gene expression of AtPUB18 and AtPUB19 during various treatments states of dormancy (datasets available through the Arabidopsis eFP Browser at BAR) can help define important criteria for studying the dormancy of the weakly dormant Columbia ecotype (Cadman et al., 2006; Finch-Savage et al., 2007). With so much cross-talk obscuring abiotic stress, hormone, and biotic stress signalling, being able to precisely pinpoint AtPUB18 and AtPUB19 involvement during seed germination and/or dormancy can be beneficial.

In general, what remains to be seen is the generation of additional and consistent abiotic-stress related phenotypes associated with combinatorial knock-out mutations and/or over-expression in AtPUB18 and AtPUB19. This thesis work has focused entirely on germination and root elongation assays; however, other parameters exist for evaluating plant sensitivity (or insensitivity) to abiotic-induced stress as well as to the hormone ABA, including stomatal aperture, water loss measurement, and electrolyte leakage to evaluate cellular membrane damage. In the meantime, refinements to multiple factors during current root elongation assays may be required to identify relevant phenotypes. Refinements to the experimental process include simultaneous treatment with more than one abiotic stress. For example, combining ABA treatment and salt stress, or combining fluridone treatment (and thus ABA-deficiency) with double knock-out seedlings with either ABA or an abiotic stress like salt, and/or readjusting assay light conditions to be lower than normal light intensities or short-day conditions could be logical options. Other considerations also consist of the creation of additional null mutations in other possible structurally or functionally redundant PUBs along with AtPUB18 and AtPUB19 double nulls (ie. creation of higher-order PUB mutants as discussed in section 4.4). Other additional mutations like crosses made with ABA mutants – either ABA-insensitive mutations like abi3, abi4, and abi5 or ABA-enhanced responsive mutations like era1 and era3 – that show pub18 pub19-distinct germination might be beneficial to reveal the sequence of events before or after PUB function during abiotic stress response.

Plants have a relatively higher number of PUBs when comparing with other eukaryotes, which has lead to speculations that this apparent gene expansion is attributable to their governance of unique plant functions. In support of PUB-ARM function extending
beyond *Arabidopsis*, two orthologs to *AtPUB18* and *AtPUB19* have been identified based on similarity clustering of ARM repeat proteins from *Arabidopsis*, rice, and *Chlamydomonas*, and they are Os08g32060 and Os09g21120 (Samuel et al., 2006). As *AtPUB19* over *AtPUB18*, Os08g32060 is a higher expressor over Os09g21120, and MAS 5.0 normalized expression for both rice genes can be seen in seed development and seedling shoot and root tissue in the Rice eFP Browser at BAR (Jain et al., 2007; Li et al., 2007). As research continues into the rice genome and its PUBs, it will be interesting to see if these same rice orthologs of *AtPUB18* and *AtPUB19* might also match the possible abiotic stress-related functions in *Arabidopsis*. With microarray data for other plant species continually becoming available, the eFP Browser in BAR has already expanded to include general tissue expression in Barley, Medicago, and Poplar as well as Poplar Drought Series and characterization of any identified PUB orthologs in these plants will become easier to access.

The work covered so far also does not address the full characterization of *AtPUB18* or *AtPUB19* proteins, including protein localization. Fluorescent protein tagging like PUB-GFP fusions would allow the visualization of *AtPUB18* or *AtPUB19* proteins in a cell and would also be suitable for judging any redistribution of subcellular localization after abiotic stress treatment, which may be informative for the mechanism of *AtPUB18*/*AtPUB19* action *in planta*. Post-treatment subcellular relocalization has been documented with other plant U-box proteins (Amador et al., 2001; Stone et al., 2003; Samuel et al., 2008) and as such has provided putative models of function for these E3 ubiquitin ligases.

Other questions to be addressed are the identities of upstream activators and downstream ubiquitination targets of *AtPUB18* and *AtPUB19*. Along with identifying the biological roles of predicted PUB proteins, understanding how these E3 ubiquitin ligases are activated is an equally important step in the elucidation of their physiological functions. For a few PUB proteins, this activation step has been uncovered and linked to receptor kinases (Kim et al., 2003; Stone et al., 2003; Samuel et al., 2008). At the same time, another critical component to understanding PUB E3 ubiquitin ligase functions is in identifying their target substrates. Interactions studies have been successfully used to identify candidates, and *in vitro* ubiquitination assays can be used
to confirm the ability of E3 ubiquitin ligases to ubiquitinate these potential substrates (Cho et al., 2006; Shen et al., 2007a; Shen et al., 2007b; Cho et al., 2008; Samuel et al., 2009). Knowledge about upstream activating kinases and downstream targeted substrates not only might bring direct focus to researching the importance and relevance of surrounding signalling components and could in itself yield insightful information into what signalling pathways AtPUB19 or AtPUB18 might regulate, it would also round out any understanding of AtPUB function.

In addition, it would be interesting to continue using microarray data to begin assessing whether AtPUB18 or AtPUB19 might function in other plant growth stages besides seed germination and seedling root elongation (Figure 6). Specifically leaf senescence appears to be a process of interest (Figure 6D) with strong AtPUB19 induction in senescent leaves, and although dark-induced senescence did not appear to be altered in single or double knock-out lines (Figure 24), senescence induced by any of the other conditions known to induce this programmed cell death process may reveal a regulatory function for AtPUB19 at this developmental time point (for example, ABA). Senescence-inducing conditions relevant to this work might be less age-related and more low-light-level-related and ABA- or abiotic stress-related, however, starting with an a quantitative assessment of senescent leaves on whole plants before and after applying such conditions might yield promising insights. Given the substantial expression of AtPUB19 in comparison to non-existent expression of AtPUB18, it will be interesting to see whether senescence assays might shed some light on possible specialized non-redundant functions of AtPUB19 separate from AtPUB18.

Finally, following up from observed emerging themes to PUB function, besides the more narrowly focused approach of full protein characterization discussed above, a more broadened view of AtPUB18 and AtPUB19 function in the context of general E3 ubiquitin ligase function could be investigated. Thus, as the number of PUB proteins associated with more defined biological functions increases, broadened observations in both their common elements and their variations in mechanistic action can further our understanding of their regulatory roles as E3 ubiquitin ligases. From the cell death seen in the AtCHIP-over-expressing plants to the alterations associated with ACRE276, AtPUB17, CMPG1, and OsSPL11 genes during the HR, the question arises as to the
mechanisms driving these phenotypes. Recently, double homozygous pub18-3 pub19-3 plants showed a decreased infection of pathogen (K. Yoshioka personal communication). It will be interesting to see, following this increased basal pathogen response, whether or not these pub18-3 and pub19-3 mutations have correspondingly increased cell death levels. While some of the PUB-associated cell death phenotypes may be genuinely related to programmed cell death or autophagy, other cases may be more related to the disruption in plant homeostasis (Love et al., 2008; Reape et al., 2008).
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APPENDICES

Appendix I. Published Material: Journal of Experimental Botany Review

Reference

Appendix II. Additional Published Material: Springer Book Chapter

## Appendix III. AGI Numbers of 64 Arabidopsis Plant U-box Proteins

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## Appendix IV. AGI Numbers of 17 UND-Containing Arabidopsis Plant U-box Proteins

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Appendix V. List of primers used for PCR screening, RT-PCR, and cloning experiments.

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<th>Original name</th>
<th>Sequence (5’- - 3’)</th>
<th>Purpose</th>
</tr>
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<tbody>
<tr>
<td>SALK-LBa1</td>
<td>LBa1</td>
<td>TTTTTCGCCCTTTGACGTTGGA</td>
<td>Left border primer for PCR screening of SALK T-DNA lines</td>
</tr>
<tr>
<td>SAIL-LB3</td>
<td>SAIL-LB3(sh2)</td>
<td>AATTTCATAACCAATCTCGATACAC</td>
<td>Left border primer for PCR screening of SAIL T-DNA lines</td>
</tr>
<tr>
<td>GK-LB</td>
<td>GK-LB (tdna)</td>
<td>CATTTGGACGTGAATGTAGACAC</td>
<td>Left border primer for PCR screening of Gabi-Kat T-DNA lines</td>
</tr>
<tr>
<td>actin3-F</td>
<td>actin-F</td>
<td>GTTGGGATGAACCAGAAGGA</td>
<td>Control for RT-PCR</td>
</tr>
<tr>
<td>actin3-R</td>
<td>actin-R</td>
<td>GAACCACCGATCCAGACACT</td>
<td>Control for RT-PCR</td>
</tr>
<tr>
<td>pub18-1-F</td>
<td>At18-634G-F3</td>
<td>TCAGAGAAACTCTCAGGCAGGA</td>
<td>PCR screening of T-DNA line</td>
</tr>
<tr>
<td>pub18-1-R</td>
<td>At18-634G-R</td>
<td>CCAGACAAGACCTATTTAAACTG</td>
<td>PCR screening of T-DNA</td>
</tr>
<tr>
<td></td>
<td>(tdna)</td>
<td></td>
<td>Screening for DEX construct</td>
</tr>
<tr>
<td>pub18-2-F</td>
<td>At18-831-F2</td>
<td>CATCGTTTTGCGCGGATTAGT</td>
<td>PCR screening of T-DNA line</td>
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<tr>
<td>pub18-2-R</td>
<td>At18-831-R2</td>
<td>GTGAACGAAACGTCCTCCTTTGGA</td>
<td>PCR screening of T-DNA line</td>
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<tr>
<td>pub18-3-F</td>
<td>At18-428gk-F</td>
<td>TGGTTGATAACGTATCCCGTGAG</td>
<td>PCR screening of T-DNA line</td>
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<td>pub18-3-R</td>
<td>At18-428gk-R</td>
<td>CTGGAACCTATGGATCATTCTTG</td>
<td>PCR screening of T-DNA line</td>
</tr>
<tr>
<td>pub19-1-F</td>
<td>At19-677-F2</td>
<td>GAGAATTGGATCAAGGGGAGACGA</td>
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<tr>
<td>Pub</td>
<td>Description</td>
<td>Primer Sequence</td>
<td>Method</td>
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<td>At19-677/791-TDNA-R</td>
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<td>PCR screening of T-DNA line</td>
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<tr>
<td>pub19-2-F</td>
<td>At19-677/791-TDNA-F</td>
<td>CTTTTTACAGATCGTTTTAGTTG</td>
<td>PCR screening of T-DNA line</td>
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<tr>
<td>pub19-2-R</td>
<td>At19-677/791-TDNA-R</td>
<td>ATTACAATAGTATAGCTCTTTGGG</td>
<td>PCR screening of T-DNA line</td>
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<tr>
<td>pub19-3-F</td>
<td>At19-099gk-F (tdna)</td>
<td>GTTATACTTCGAGGCATTGATGTT</td>
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<tr>
<td>pub19-3-R</td>
<td>At19-099gk-R (tdna)</td>
<td>CCCCAGACGGAGCACTGAT</td>
<td>PCR screening of T-DNA line</td>
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<td>AtPUB18-promoter-F</td>
<td>At18pr-Pstl-p-2029-F (clon)</td>
<td>gcgtgcag</td>
<td>Cloning of AtPUB18 promoter with Pstl site</td>
</tr>
<tr>
<td>AtPUB18-promoter-R</td>
<td>At18pr-Xmal-R (clon)</td>
<td>gcgcgcggg</td>
<td>Cloning of AtPUB18 promoter with Xmal site</td>
</tr>
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<td>AtPUB19-promoter-F</td>
<td>At19pr-F (cloning)</td>
<td>ATAAAATAACTCAAAAGCTGAGTTG</td>
<td>Cloning of AtPUB19 promoter</td>
</tr>
<tr>
<td>AtPUB19-promoter-R</td>
<td>At19pr-R (cloning)</td>
<td>TTAATAAGATAAGATATGAGTGAAAA</td>
<td>Cloning of AtPUB19 promoter</td>
</tr>
<tr>
<td>AtPUB18-DEX-F</td>
<td>At18-Xhol-F (clon)</td>
<td>gcgtgcgaga</td>
<td>Cloning of AtPUB18 with Xhol site for DEX construct</td>
</tr>
<tr>
<td>AtPUB18-DEX-R</td>
<td>At18-Xhol-R (clon)</td>
<td>gcgtgcgaga</td>
<td>Cloning of AtPUB18 with Xhol site for DEX construct</td>
</tr>
<tr>
<td>DEX::At18-F</td>
<td>pTA7002-F</td>
<td>ACCCTTCTCTATATAAGGAAGT</td>
<td>PCR screening for DEX construct</td>
</tr>
<tr>
<td>DEX::At18-R</td>
<td>At18-634G-R (tdna)</td>
<td>CCAGACAAGACCTATTTAAAACGT</td>
<td>PCR screening for DEX construct</td>
</tr>
</tbody>
</table>

**CAPITALIZED** = annealing bases  **Underlined** = restriction enzyme site
Appendix VI. Vector map for pTA7001 (DEX inducible promoter) with AtPUB18

pTA7001 information as provided from Dr. Brian Ellis lab at the University of British Columbia.

pTA7001 + AtPUB18 *

Sequence (incomplete) of T-DNA region of vector pTA7001 (empty) as follows:

1-39: pTiPOST37 from pBI101 (RB=1-25)
47-858: 35S promoter from pBI221 (TATA=813-816)
867-1097: GAL4 (aa1-77)
1117-1340: VP16 (aa413-490)
1347-2180: ratGR (aa519-795)
2207-2764: pea rbcs-E9 terminator
2780-3112: NOS promoter from pBI101
3120-4145: hygromycin phosphotransferase (p2929-2931)
4147-4399**: NOS terminator from pBI101
4893-4423**: pea rbcs-3A terminator
4941-4894**: cloning sites SpeI & XhoI
4995-4942**: 35S promoter TATA region (TATA=4980-4977)
5197-4996**: 6xGAL4 UAS
5198-5357: M13mp19 EcoRI-Haell fragment from PBI101
5358-5862: pTiPOST37 from PBI101 (LB=5838-5862)

** indicates reversed orientation from that of pTA7002 vector

AtPUB18 was cloned using the XhoI insertion site. Proper orientation was confirmed.
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**Figure 2:** Gene expression microarray profiles for *AtPUB* genes in *Arabidopsis* from the *AtGenExpress Consortium* data sets under a range of conditions and treatments.

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http://jxb.oxfordjournals.org/cgi/content/abstract/60/4/1109

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**Figure 2.** Phylogenies of U-box and UND protein sequences.

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