Title of Dissertation: BOLT, AN AP2/ERF TRANSCRIPTION FACTOR, REGULATES ABIOTIC STRESS AND DEFENSE RESPONSES IN ARABIDOPSIS THALIANA

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Biotic and abiotic stresses negatively affect plant growth and development, hence decrease productivity. Many AP2/ERF family transcription factors in plants have important roles in stress response signaling although most have not yet been studied. Here I show that expression of an Arabidopsis thaliana AP2/ERF family member, which I call BOLT, is regulated by a MAPK pathway that includes MEKK1, M KK1, M KK2, and MPK4, and has roles in both biotic and abiotic stress response as well influencing growth and development. In this thesis, I examined BOLT’s gene expression pattern and protein localization, using GUS and YFP reporter genes respectively, measured its expression in response to biotic and abiotic stress and plant hormones using RT-qPCR, examined phenotypes by generating overexpressing and RNAi lines, and analyzed its effect on downstream gene expression using a microarray at time points after inducing
BOLT expression. I found that BOLT is expressed in various plant tissues and the protein localizes to nuclear bodies as demonstrated in onion epidermal cells. Knockdown (RNAi) plants exhibit greater drought tolerance and are larger than wild type under low light conditions, while the overexpressors exhibit a dramatic early flowering phenotype and are small and weak under low light. Gene expression analysis suggests BOLT regulates genes involved in photosynthesis, hormone biosynthesis and signaling, and defense, many of which are also regulated in the MAPK pathway. Increased BOLT expression downregulates two discreet systems, cyclic electron flow and glycine cleavage, components of photosynthesis and photorespiration, respectively, which are two systems that are important under low light conditions. Taking these results together, I conclude that BOLT functions downstream of a stress responsive MAPK pathway and regulates a variety of growth- and stress-related genes necessary to balance growth and defense in response to biotic or abiotic stresses, or low light conditions.
BOLT, AN AP2/ERF TRANSCRIPTION FACTOR, REGULATES ABIOTIC STRESS AND DEFENSE RESPONSES IN ARABIODPSIS THALIANA

by

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Dedication

To my three bolts, Sheila, William, and Hanna.
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Dr. Jorge G.T. Zañudo analyzed the microarray data and constructed the co-expression network. I would like to thank him for his many patient explanations. Dr. Peter Morris kindly provided the mkk mutant seeds that were invaluable to this project.

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List of Abbreviations

ABA, Abscisic Acid
ABI3, ABA insensitive 3, At3g24650
ACS7, Amino Cyclopropane Synthase 7, At4g26200
amiRNA, Artificial Micro RNA
AP2, APETALA2
BBX32, B-Box Domain Protein 32, At3g21150
BiFC, Bimolecular Fluorescence Complementation
BOLT, At1g01250
CBF1/DREB1B, C-Repeat-Binding Factor 3, At4g25490
CBF2/DREB1C, C-Repeat-Binding Factor 3 At4g25470
CBF3/DREB1A, C-Repeat-Binding Factor 3 At4g25480
CBF4/DREB1D, C-Repeat-Binding Factor 4, At5g51990
cDNA, Complementary DNA
CEF, Cyclic Electron Flow
chapter 4 genes related to flowering and circadian
ChIP-seq, Chromatin Immunoprecipitation-Sequencing
CNGC12, Cyclic Nucleotide-Gated Channel 12, At1g46450
CNGC4, Cyclic Nucleotide-Gated Channel 4, At5g54240
Co-IP, Co-Immunoprecipitation
COR15A, Cold-Regulated 15A, At2g42540
CRR3, Chlororespiratory Reduction 3, At3G01590
CRR7, Chlororespiratory Reduction 7, At5G39210
DAVID, Database for Annotation, Visualization, and Integrated Discovery
DDF1, Dwarf and Delayed Flowering 1, At1g12610
DDF2, Dwarf and Delayed Flowering 2, At1g63030
EBI, European Bioinformatics Institute
EDF1, Ethylene Response DNA Binding Factor 1, At1g25560
EDF2, Ethylene Response DNA Binding Factor 2, At1g68840
EDF3, Ethylene Response DNA Binding Factor 3, At3g25730
EDF4, Ethylene Response DNA Binding Factor 4, At1g13260
EIN3, Ethylene-Insensitive 3, At3g20770
ERF, Ethylene Response Factor
ERF022, Ethylene Response Factor 022, At1g33760
ERF1, Ethylene Response Factor 1, At3g23240
ERF104, Ethylene Responsive Factor 104, At5g61600
ERK, Extracellular Signal-Regulated Kinase
ESE2, Ethylene and Salt Inducible 2, At3g25820
FDR, False Discovery Rate
flg22, Flagellin Peptide
FUF1, Forever Young Upregulating Factor, At1g71450
FUS3, FUSCA3, At3g26790
FYF, Forever Young Flower, At5g62165
GDC, Glycine Decarboxylase Complex
GFP, Green Fluorescent Protein
GO, Gene Ontology
GOX1, Glycolate Oxidase 1, At3g14420
GOX2, Glycolate Oxidase 2, At4g14415
HPR1, Hydroxypyruvate Reductase, At1g68010
HRD, Hardy, At2g36450
HY5, Elongated Hypocotyl 5, At5g11260
LEC1, Leafy Cotyledon 1, At1g21970
LEC2, Leafy Cotyledon 2, At1g28300
LHY, Late Elongated Hypocotyl 1, At1g01060
MAP, Mitogen Activated Protein
MAPK, Mitogen Activated Protein Kinase
MEKK1, MAPK/ERK Kinase Kinase 1, At4g08500
MKK1, Mitogen Activated Protein Kinase Kinase 1,
MKK2, Mitogen Activated Protein Kinase Kinase 2, At4g29810
MKS1, Mitogen Activate Protein Kinase Substrate 1, At3g18690
MPK4, Mitogen Activated Protein Kinase 4, At4g01370
NASC, Nottingham Arabidopsis Stock Centre
NCBI, National Center for Biotechnology Information
NDH, NAD(P)H Dehydrogenase
NDHA, NAD(P)H Dehydrogenase A, Atcg01100
NDHF, NAD(P)H Dehydrogenase F, Atcg01010
NDHM, NAD(P)H Dehydrogenase M, At4g37925
NDHN, NAD(P)H Dehydrogenase N, At5G58260
NDHO, NAD(P)H Dehydrogenase O, At1G74880
NDHT, NAD(P)H Dehydrogenase T, At4G09350
NF-YA4, Nuclear Factor Y, subunit A4, At2g34720
NF-YA7, Nuclear Factor Y, subunit A7, At1g30500
NF-YB2, Nuclear Factor Y, subunit B2, At5g47640
NLS, Nuclear Localization Signal
NPR1, Nonexpressor of PR Genes, At1g64280
OE, Overexpressing
OPR3, Oxophytodienoate-reductase 3, At2g06050
PAD4, Phytoalexin Deficient 4, At3g52430
PNSB1, Photosynthetic NDH Subcomplex B 1, At1G15980
PNSB2, Photosynthetic NDH Subcomplex B 2, At1G64770
PNSL1, Photosynthetic NDH Subcomplex L 1, At2G39470
PNSL2, Photosynthetic NDH Subcomplex L 2, At1G14150
PNSL3, Photosynthetic NDH Subcomplex L 3, At3G01440
PNSL4, Photosynthetic NDH Subcomplex L 4, At4G39710
PQ, Plastoquinone
PR1, Pathogenesis-related protein 1, At2g14610
PSI, Photosystem I
PSII, Photosystem II
qPCR, Quantitative Polymerase Chain Reaction
q-value, FDR-adjusted p-value
RD29A, Responsive to Desicication 29A, At5g52310
RNA-seq, RNA sequencing
RNA-seq, RNA-Sequencing
RNAi, RNA Interference
ROS, Reactive Oxygen Species
RQI, RNA Quality Indicator
RVE8, Reveille 8, At3g09600
SA, Salicylic Acid
SID2, SA Induction Deficient 2, At1g74710
TAIR, The Arabidopsis Information Resource
TINY, At5g25810
TINY2, At5g11590
YFP, Yellow Fluorescent Protein
ZTL, Zeitlupe, At5g57360
Chapter 1: Abiotic and biotic stress signaling leads to transcription factor activity

Changing environmental conditions together with increasing world population puts pressure on society to be able to produce sufficient crop plants. Diversity of plant species, changes in distribution and community associations are also concerns as plants move and adapt to changes at different rates. In addition to impacting food production, these developments affect air quality, soil erosion, carbon sequestration, and further raise global temperatures (Millar et al. 2007; Nearing et al. 2004; Kelly & Goulden 2008). Our understanding of stress responses in plants allows us to more intelligently breed and engineer crop plants that can better tolerate current and future growing conditions as well as to manage forests and other ecosystems to minimize the disruptions these global changes are inducing. Plants under stress use response mechanisms to restrict their growth, flower early, or otherwise adjust their growth and development under hostile conditions resulting in outcomes that can reduce crop yield. The ability to generate plants well suited to their environments and able to successfully deal with stress can help ensure food security for the future.

Environmental stresses impact myriad aspects of plant growth, development, and reproduction. Plants, being sessile organisms, cannot avoid adversity by changing location to find water, avoid heat or cold, or escape insects, so must have systems to maintain homeostasis in the face of harsh conditions and pathogen attack. To do this, plants have developed wider ranging defense mechanisms than animals, which are able to move about. Plants are subject to abiotic stresses including excess or insufficient water or
light, extremes of temperature, salinity, heavy metals, mechanical wounding, and others. Biotic stresses include bacteria, fungus, parasites, and insects. These challenges require well-orchestrated responses that include sensing, signaling, and changes to gene expression in response to numerous and multiple stresses. The coordination and control of gene expression (transcription regulation) is critical to the successful survival and reproduction of the plant. I am interested in understanding changes in gene expression that are responsible for the various responses we see in plants subjected to different growth environments. These changes are mediated, in part, by factors that enhance or inhibit transcription in response to biotic and abiotic stresses perceived by the plant.

**Stress perception and signal transduction by a MAPK pathway**

The sensing of stress signals and their transduction into appropriate responses is crucial for the adaptation and survival of plants. Plant cells require mechanisms to perceive changes in the environment and signal those changes to organelles that can produce the necessary responses such as the synthesis of hormones, antioxidants, defense chemicals, as well as gene expression to continue the response and adapt to a new condition. Cells respond to stress through a wide array of cell surface receptors such as histidine kinases, receptor-like kinases, and NDP kinases (Moon et al. 2003; Osakabe et al. 2013; Shiu & Bleecker 2001). These receptors are activated by specific aspects of stress, one type of stress potentially activating a number of different receptors based on degree and coincident stresses. It has also been shown that there is considerable cross-talk among the receptors allowing further specificity to the information transduced (Osakabe et al. 2013; Siddhi K Jalmi 2015).
Perception of both biotic and abiotic stresses results in the immediate production of reactive oxygen species (ROS), major stress-signaling molecules (Rejeb et al. 2014). ROS are signaling elements common to both biotic and abiotic stress responses that are generated at the plasma membrane as well as in organelles, predominantly chloroplasts, mitochondria, and peroxisomes (Apel & Hirt 2004; Carvalho 2008; Mittler 2002; Rahikainen et al. 2016; Foyer & Noctor 2005). ROS have different effects in stress conditions depending on the types and levels of accumulation, the mechanisms of which are not well understood (Mittler 2002; Dat et al. 2000). Low levels of ROS function in stress signaling and in trigger defense/acclimation responses, whereas high levels can result in cell death (Dat et al. 2000; Mittler 2002). ROS activate mitogen activated protein kinase (MAPK) pathways that are specific to the types and magnitude of the stresses perceived (Mittler et al. 2011). MAPK pathways control stress responses and link upstream receptors to downstream targets (Rejeb et al. 2014). MAPK pathways are also activated by direct interaction with receptors (Moon et al. 2003; Mizuno & Yamashino 2010). It is clear that ROS signaling and MAPK cascades have important roles in gene expression under stress conditions, however the signal-specific ROS sensing and transducing mechanisms are unknown (Mittler et al. 2011).

MAPK pathways consist of highly conserved protein modules, ubiquitous in eukaryotes, that act in signaling cascades (Cristina et al. 2010). Each cascade includes a minimum of three protein kinases, a MAP3K (MEKK), a MAP2K (MEK or MKK), and a MAPK (MPK) that are sequentially activated by phosphorylation. In the Arabidopsis genome 80 MAP3Ks, 10 MAP2Ks, and 23 MAPKs have been identified suggesting the possibility of a very large number of combinations (Taj et al. 2010; Hamel et al. 2006).
Distinct MEKK/MKK/MPK modules have been identified that have overlapping functions in abiotic stress response, defense and immunity, and development (Meng & S. Zhang 2013; Cargnello & Roux 2011; Leissing et al. 2016).

MAPK cascades have important roles in abiotic and biotic stress responses as well as in stress-induced growth and development (Colcombet & Hirt 2008). Activation of MAPK cascades is one of the earliest signaling events after perception of stress by the plant (Meng & S. Zhang 2013). MAPKs are involved in signaling multiple defense responses including the biosynthesis/signaling of plant stress/defense hormones and other secondary metabolites, generation of ROS, stomatal closure, cell wall strengthening, and gene activation (Meng & S. Zhang 2013). Once activated MAPKs go on to phosphorylate target substrates (Meng & S. Zhang 2013). Substantial crosstalk between MAPKs in different stress-induced pathways has been shown, although the ultimate signal response of a cascade is known to be specific for a particular stimulus in the activation of downstream targets (Siddhi K Jalmi 2015). MPK substrates have been shown to be enriched in transcription factors involved in the regulation of development, defense, and stress responses (Popescu et al. 2009).

**Stress induced transcription regulation**

Numerous transcription factors are known to have roles in stress responses in Arabidopsis (Licausi et al. 2013; Baldoni et al. 2015; Bakshi & Oelmüller 2014; Nuruzzaman & Sharoni 2015). Transcription factors function, together with other factors, to activate or repress expression of genes encoding downstream regulatory and structural proteins, enzymes, and other functional gene products in response to exogenous and
endogenous signals including stress, growth, and development cues. These proteins bind specific DNA sequences, and it is on the basis of this binding domain that they are classified into gene families (Riechmann et al. 2000). Transcription regulation is complex, sophisticated, and specific in terms of cell-type and developmental stage, as well as to prevailing environmental conditions.

We can see by perusing the publically available databases of genome-wide transcriptome analyses that many transcription factors have roles in regulating a wide variety of stresses (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011). Transcription factors from every major family have been shown to have roles in stress responses (Licausi et al. 2013; Baldoni et al. 2015; Bakshi & Oelmüller 2014; Nuruzzaman & Sharoni 2015). A single transcription factor may respond to several stresses and the response of one transcription factor can change based on the level of stress as well as the presence of other stresses that are concurrent eliciting, a total response that is different from each individual response (Voelckel & Baldwin 2004).

As a result of the complete sequencing of the *Arabidopsis* genome and the development of such bioinformatics tools as BLAST, it has become much less complicated to identify genes/proteins that share common motifs. The identification and categorization of large transcription factor families followed these advancements. To date over two thousand transcription factors have been identified in Arabidopsis and classified into over fifty families by their common DNA binding domains (J. Jin et al. 2014; J. Jin et al. 2015). More than nine hundred of these genes are in the six largest families: AP2/ERF, bHLH, bZIP, MYB, NAC, and WRKY (J. Jin et al. 2015; J. Jin et al. 2014). Transcription factors from all of these families have been shown to regulate biotic and
abiotic stress responses as well as growth and development, however only a fraction of them have been characterized (Singh et al. 2002; Nakano et al. 2006; Carretero-Paulet et al. 2010; Ambawat et al. 2013). For example, although 146 AP2/ERF transcription factors have been identified, only about 50% are mentioned in a publication, and fewer still have been the focus of a study.

**The AP2/ERF family of transcription factors**

In 1994, Jofuku et al. identified an essential amino acid motif in APETELA2 (AP2), a transcription factor that has a role in flower and seed development in *Arabidopsis* (Jofuku et al. 1994). Around the same time Ohme-Takagi identified an ethylene responsive DNA-binding domain and cloned and characterized several ethylene responsive binding proteins (EREBPs) that specifically interacted with that DNA (Ohme-Takagi & Shinshi 1995). This domain was the same as the motif in APETELA2, hence the various nomenclature to refer to the same domain. The AP2 domain, as it is now commonly referred to, is a conserved DNA-binding element consisting of 60-70 amino acids. The three-dimensional structure of the AP2 domain of ERF1 (At3g23240) was solved by NMR (Allen et al. 1998). The structure consists of a three-stranded β-sheet and one α-helix (Fig1.1). Soon afterwards, DNA-binding proteins from tobacco that had domains closely related to the AP2 were identified. They were shown to interact with an ethylene responsive promoter sequence, and were referred to as AP2/EREBP (ethylene-responsive element binding proteins) (Ohme-Takagi & Shinshi 1995).

A short time later, Riechmann et al described the AP2/EREBP, now referred to as the AP2/ERF (ethylene response factor), family of plant transcription factors as a large,
multigene family with 144 members, whose functions include developmental processes as well as response to biotic and abiotic stresses in many plant species (Riechmann & Meyerowitz 1998; Riechmann et al. 2000). The AP2/ERF family is one of the largest gene families in plants and initially it was thought to be plant specific. AP2 domains are not found in animal genes, but Magnani et al demonstrated that homologs are present in a cyanobacterium, a ciliate, and several viruses (Magnani et al. 2004). The group also demonstrated functional conservation between the prokaryotic and plant AP2 domains and hypothesized a horizontal transfer of an AP2 domain-containing endonuclease protein from bacteria or viruses into plants as the origin of the AP2/ERF transcription factor family in plants. The original nomenclature of Ethylene-response and Ethylene-binding has been retained although AP2/ERF are not universally responsive to ethylene (Licausi et al. 2013).
Figure 1.1 Model of the AP2 binding domain.

NMR structure of the GCC-BOX binding domain of Arabidopsis ERF1 (green) complexed with its target DNA. Based on PDB 1GCC (Allen et al. 1998).
Sakuma et al. studied the DNA-binding specificity of the AP2/ERF domain in *Arabidopsis* (Sakuma et al. 2002). The authors focused on DREB (dehydration-responsive element binding) proteins, a subset of AP2/ERF transcription factors that bind a DRE sequence (dehydration responsive element), but also undertook an amino acid based sequence analysis of the now 145 AP2/ERF proteins. As a result of the study the authors classified the proteins in Arabidopsis into groups on the basis of the number of AP2 repetitions and amino acid sequence of the individual AP2 domains. The classification consisted four subfamilies including: DREB (56 genes) and ERF (65 genes) each having a single AP2 domain, AP2 (18 genes) with 2 AP2 domains, and RAV (6 genes) having one AP2 domain as well as one B3 DNA-binding domain. Using binding and expression analyses, the group found that both DREB1A and DREB2A bind the DRE element and that *DREB1A* expression is induced by cold temperature in the leaf and root, and to a lesser extent by salt in the root, while *DREB2A* expression is highly induced by salt in the root, as well as dehydration and ABA in the leaf and root (Sakuma et al. 2002).

Several years later, taking advantage of the increased availability of genomic data, Nakano et al. (2006) carried out a comprehensive computational analysis of the AP2/ERF family of transcription factors that considered the entire amino acid sequences of the AP2/ERF genes in Arabidopsis, grouping them based not only on the similarities or differences of the AP2 domains, but also based on the rest of the proteins’ sequences (Nakano et al. 2006). The analysis resulted in minor changes at the subfamily level, but within the subfamilies, genes were recategorized into different groups based on numerous shared conserved motifs, most of which have no known function. In the same paper, the authors summarized the functions of AP2/ERF genes that had been reported to that point.
Using overexpression, knockout mutants, and activation tagging, researchers reported a function for twenty-eight AP2/ERF genes in *Arabidopsis*, tobacco, rice, and other species. These functions included abiotic stress, response to hormones, disease resistance, wax accumulation, organ identity, and leaf petiole development indicating that AP2/ERF transcription factors may have wide roles in plant development and stress responses.

In Nakano et al’s analysis, At1G01250, a gene which I have named *BOLT* due to an early bolting phenotype described in Chapter 3, is classified as a Group III AP2/ERF transcription factor, a DREB, along with twenty-two other genes (Fig. 1.2). Group III, along with Groups I, II, and IV correspond to Sakuma’s DREB subfamily and are often referred to as such. The ERF subfamily comprises Nakano et al’s Groups V – X (Sakuma et al. 2002; Nakano et al. 2006). It is interesting to note that fewer than twenty of the 146 AP2/ERF genes contain an intron, none in Group III, and that the positions of the introns are conserved within the groups. An alignment highlights the similarities in the amino acid sequences of the AP2 domain in Group III and the lack of similarity among the sequences that are outside the AP2 domain (Figs. 1.3 and 1.4).
Figure 1.2 Phylogenetic relationships among AP2/ERF transcription factors

AP2/ERF transcription factors in Arabidopsis thaliana. The AP2 domain and conserved motifs are indicated by colored boxes. BOLT is in Group III, outlined in red (Nakano et al. 2006).
Figure 1.3 Alignment of AP2 domains of Group III AP2/ERF transcription factors

Blue arrows indicate the extent of the AP2 domain. β-sheets and α-helix shown in green. BOLT is outlined in red. Known functions of these genes are described in the following section.
Figure 1.4  AP2/ERF Group III entire protein sequences alignment

The AP2 domain is well conserved as are several motifs immediately following, however, the remainder of the sequences are divergent.
Broadly, a number of DREB proteins, including CBF1 and CBF3 of Group III, have been shown to bind a DRE element which is associated with dehydration, cold, and ABA responsive genes (Stockinger et al. 1997), and some ERF proteins bind the GCC box often found in the promoter regions of ethylene, pathogen, and wounding responsive genes (Ohme-Takagi & Shinshi 1995). This is a generalization though, and recently, members of both subfamilies have been shown to bind both elements as well as additional DNA motifs (Sun et al. 2008; Dietz et al. 2010; Shaikhali et al. 2008). TINY, another Group III gene, for instance was shown to bind the DRE and the ERE with similar affinity (Sun et al. 2008).

**Group III transcription factors and stress responses**

Since BOLT is classified as a Group III AP2/ERF transcription factor, it is worth reviewing the current understanding in the field as it pertains to these genes. In the case of the twenty-three Group III transcription factors, most of the work is more recent than that discussed above, and is focused on elucidating gene expression rather than DNA binding. Four genes, *CBF1/DREB1B, CBF2/DREB1C, CBF3/DREB1A,* and *CBF4/DREB1D,* known to respond to cold, salt, and dehydration, recently were shown to have a role in integrating cold signaling with the circadian clock (Chow et al. 2014; Haake et al. 2002). Five additional genes, *HRD, DDF1, DDF2, TINY,* and *TINY2* were also upregulated in response to salt, and dehydration, with all except *HRD* also responsive to cold (Abogadallah et al. 2011; Karaba et al. 2007; Magome et al. 2004; Magome et al. 2008; Kang et al. 2011; Hong et al. 2013; Sun et al. 2008; Wei et al. 2005). A study of *ESE2* showed it binds to promoters of *RD29A* and *COR15A,* which are
known to respond to salt, dehydration, and cold temperature, and it is genetically
downstream of EIN3, a known ethylene signaling component (L. Zhang et al. 2011).
These examples indicate roles for AP2/ERF transcription factors in responding to a
number of abiotic stresses.

Group III transcription factors have been shown to respond to biotic stresses as
well as abiotic stresses. HRD, in addition to responding to salt and dehydration is also
activated by infection with downy mildew (Huibers et al. 2009). The same study showed
Group III genes At1g63040, At2g36450, and At5g52020 also upregulated by infection
with downy mildew (Huibers et al. 2009).

In addition to biotic and abiotic stress roles, different or additional roles have been
suggested for Group III transcription factors. For example, FUF1 regulates flower
senescence/abscission through FYF by suppressing EDF1/2/3/4, ERF022 regulates
ethylene biosynthesis through ACS7 and ethylene signaling through ERF1 and ETR1,
negatively controlling ethylene content and perception, and At3g60490 is directly
downregulated by redox signals from the photosynthetic electron transport chain (W.-H.
Chen et al. 2015; Nowak et al. 2014; Fey et al. 2005). Further results in the study of TINY
showed that overexpression resulted in a dwarf phenotype under normal growth
conditions as well as a partial constitutive triple response in a 3-day-old dark-grown
seedlings, implying TINY could have a role in ethylene response (Sun et al. 2008).

We must keep in mind that you don’t find what you don’t look for, for instance in
order to show a gene is responsive to a particular stress, experiments usually must be
designed to show that. Development genes are even more tricky as they are expressed on
a schedule as well as often responding to stress, which makes the plant’s age and
developmental stage crucial in designing experiments and interpreting results.

Additional information about possible functions can be gleaned from searching
curated microarray and RNA-seq data by such institutions such as European
Bioinformatics Institute (EBI’s Expression Atlas) and companies such as Genevestigator
(Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011)
(https://genevestigator.com). These groups make expression data for thousands of
experiments available and easily searchable. While response to stress is not confirmation
of function, these results suggest many of these proteins may have multiple roles in
diverse stress, growth, and development. For instance, all four CBF genes that are known
for abiotic stress response were downregulated by fungus, nematode and other pathogenic
treatments in experiments in these collections. FUF1 is implicated in flower development
in the literature, but is also shown to be upregulated in cold and salt conditions. This
further suggests that these Group III genes can respond to different types of stresses, and
adding an additional level of complexity, it has also been shown that transcriptome
changes in response to double stresses were not predicted from the responses to single
stress treatments (Rasmussen et al. 2013).

Here, I will examine the role of BOLT in stress responses and show that BOLT
expression changes not only in response to abiotic stress, but also in response to biotic
stress. Then I will show that overexpressing BOLT results in an early-flowering
phenotype and a leaf morphology that is different from wild type. In addition, I will
present evidence that photoperiod and particularly light intensity affects plant growth in
the overexpressing lines compared to wild type, suggesting a possible role for BOLT in
photosynthesis under certain conditions. Finally, I will discuss the results of a genome-wide expression analysis comparing wild type expression to induced overexpression in the 24 hours after induction, and show the results of a co-expression analysis that supports directed expression regulation emanating from BOLT.
Chapter 2: *BOLT* is expressed in various plant tissues and responds to abiotic and biotic stresses

**Introduction**

When I joined the Kwak lab one of the projects I was given was to determine the function of At1G01250, a member of the AP2/ERF family of transcription factors that had not been characterized. I subsequently named the gene *BOLT* as a result of an early bolting phenotype described in Chapter 3. The reason we were interested in the gene initially was because a microarray carried out in Julian Schroeder’s lab, where Dr. Kwak had been a postdoc, showed its expression to be higher in guard cells than in mesophyll cells. The objective of that microarray experiment was to isolate promoter candidates, for the purpose of manipulating gene expression highly and specifically in guard cells. *BOLT* was not identified in the resulting paper (Y. Yang et al. 2008), but was thought to have a potential role in guard cell function or development because of its higher expression in guard cells as compared to mesophyll cells and for this reason we undertook this study.

Although BOLT looked like a promising candidate as a guard cell specific or preferential transcription regulator, upon closer inspection of the Schroeder lab’s microarray data, the p-values assigned the BOLT samples were much too high to be statistically significant, and as described below, I subsequently found that *BOLT* is expressed in various tissues throughout the plant including both guard cells and mesophyll cells. The high p-values pertained only to the *BOLT* samples, which were not included in, thus don’t impact the Yang (2008) publication.
As described in Chapter 1, BOLT is in the Group III AP2/ERF transcription factor family, and that of the Group III genes that have been studied there are examples of stress responsive genes as well as genes with roles in development. It is clear too, that many of the Group III transcription factors have roles in more than one stress or type of stress, responding to dehydration (abiotic) and fungus (biotic) for instance in the case of HRD, or to cold and chitin (main component of insect shells and cell walls of some fungi) in the case of TINY (Huibers et al. 2009; Karaba et al. 2007; Sun et al. 2008). FUF1 is upregulated during flower development, and was also shown to respond to cold, salt, and nematode (a parasitic roundworm) (W.-H. Chen et al. 2015).

The Chapter 1 review of the literature suggests that BOLT could respond to one or more abiotic or biotic stresses. In order to understand BOLT’s potential role in stress response, I sought to answer several key questions. First, since the results suggesting that BOLT was preferentially expressed in guard cells were not reliable, I wanted to know where in the plant BOLT is expressed. Second, I wanted to determine the sub-cellular localization of the BOLT protein. Being a likely transcription factor, BOLT might localize to the nucleus, or it could remain in the cytoplasm and be transported to the nucleus under particular conditions. The sub-cellular localization could suggest a mechanism for how BOLT functions. A third important question is under what conditions BOLT’s expression changes. The answers to these questions will helped direct further investigation into BOLT’s role as a putative transcription factor.
Results

*BOLT is expressed in various tissues and the protein localizes to nuclear bodies*

An initial question when investigating a new gene/protein is where in the plant and where in the cell does it function. These two questions can be addressed in part by determining the expression pattern of the gene and the sub-cellular localization of the protein. BOLT was originally thought to be specific or preferential to guard cells and I sought to confirm this expression pattern. Understanding where and how much *BOLT* is expressed in *Arabidopsis* may suggest an area of functionality so I performed two experiments to determine the expression pattern. First I made transgenic plants that express a *BOLT* promoter::GUS fusion reporter driven by the *BOLT* promoter region including 1,666 base pairs upstream from the translation start site. I stained and examined twenty-four independent lines. Using the 1,666 base-pair promoter fragment would obviously not include potential cis-elements outside that range.

Expression per GUS staining was clear in leaves, flowers, stems, embryos of mature green seeds, and vasculature. In the root and stems, staining was in the vasculature only. In the leaves, staining was throughout the leaf in young leaves as well as in the vascular tissue. Staining was much darker in emerging leaves than in expanded leaves. In expanded leaves staining was visible in guard cells, but it was not as dark as in the vasculature and other parts of the plant. Flowers and seeds stained darker than all other parts of the plants. In the root, staining was evident in the root tip, but not immediately behind the root tip. There was staining in lateral roots, but none in root hairs (Fig. 2.1).
In the second experiment designed to identify in which tissues $BOLT$ is expressed, I isolated RNA from flower, stem, leaf, and root samples of wild-type *Arabidopsis*, transcribed cDNA, and performed Real Time Quantitative Polymerase Chain Reaction (RT-qPCR) to measure the relative expression in those tissues. Here too, $BOLT$ was expressed in all tissues sampled, with higher expression in the aerial parts of the plant than in the roots. Of the tissues I tested, expression was highest in flowers (Fig 2.1). I concluded from these experiments that $BOLT$ is not only expressed in guard cells, but is expressed to different degrees widely in the plant.

$BOLT$ is a putative transcription factor, so the subcellular localization of the protein is an important factor in its activity. I was interested to know if $BOLT$ localizes to the nucleus or cytoplasm under normal growing conditions. First I consulted nuclear localization prediction software to see if there are any predicted nuclear localization signals (NLS) in the amino acid sequence. I used three predictors which all identified one potential NLS sequence and one predicted a second potential NLS sequence (Kosugi et al. 2009; Brameier et al. 2007). The site predicted by all three has a RKRR sequence, which is a known plant NLS signal (Moes et al. 2008).
Figure 2.1 Tissue expression of promoter $BOLT::GUS$

Staining indicates $BOLT$ expression in a variety of tissues including vascular, embryo, root and inflorescence. A 1666 bp fragment of DNA upstream from $BOLT$’s transcription start site was fused to a GUS reporter gene, and $BOLT$ promoter::GUS transgenic plants stained with x-gluc. A, B, and D, ten-day-old seedling, leaf – including guard cells, and root. C, mature green seeds, E, inflorescence. F, $BOLT$ expression in flower, leaf, and root as measured using RT-qPCR. Both $\beta$-actin and UBI10 were used as reference genes. Data represents three biological replicates and error bars show standard error of the mean.
In order to determine where the BOLT protein is localized in the cell, I made transgenic *Arabidopsis* lines expressing *BOLT* with either an N-terminal or a C-terminal yellow fluorescent protein (YFP) reporter driven by the cauliflower mosaic virus constitutive (CaMV35S) promoter. I isolated more than a dozen homozygous, independent lines for each construct and examined the roots using a Zeiss AxioObserver inverted fluorescence microscope to confirm and assess the fluorescence in each line. In the transgenic plants, BOLT was clearly localized to the nucleus in *Arabidopsis* roots (Fig. 2.2). To obtain additional information about BOLT’s localization, I decided to transiently express the 35S::*BOLT*:YFP construct in onion epidermis because onion cells are large, transparent, and in easily manipulated layers. So, using a gene gun, I bombarded onion epidermis with gold particles coated with vectors containing each of the constructs (C-terminal, and N-terminal). In the onion epidermal cells too, BOLT was localized to the nucleus (Fig 2.2). Interestingly, inside the nucleus, the YFP signal was concentrated in nuclear bodies (Fig. 2.2). Both constructs resulted in similar localization and I continued experiments using the C-terminal lines. We can conclude from the in vivo experiment using transgenic *Arabidopsis* plants, the transient expression in onion epidermal cells, and the NLS in silico sequence information, that BOLT localizes to the nucleus and further that it may localize to sub-nuclear bodies under some conditions.

**BOLT expression changes in response to abiotic and biotic stress treatments**

*BOLT* is implicated in stress response as a result of its classification as an AP2/ERF transcription factor in the Group III subfamily, thus I was interested to see how its expression changed in response to various abiotic and biotic stresses. To do this I
subjected ten-day-old plate-grown seedlings to abscisic acid (ABA), dehydration, cold temperature, wounding, flg22 peptide (a bacterial peptide epitope commonly used to trigger immune responses in plants), salicylic acid (SA), or Methyl Jasmonate (MeJA) and sampled whole seedlings several times over the course of 24 hours. I isolated RNA, transcribed cDNA and performed RT-qPCR experiments to compare \textit{BOLT}'s expression in stressed and unstressed wild-type plants. The results showed \textit{BOLT} expression increased when the plants were subjected to ABA, dehydration, cold temperature, wounding, and the flg22 peptide. and decreased with the application of SA and MeJA (Fig. 2.3). ABA, SA, and MeJa are each associated with a variety of stress responses, as well as other signaling activity in \textit{Arabidopsis} (Murphy, 2015). These results indicated that \textit{BOLT} expression changes in response to certain abiotic and biotic stresses, as well as in response to hormones associated with stress response, consistent with its classification as an AP2/ERF Group III transcription factor.
Figure 2.2  35S::BOLT::YFP expressed in onion epidermis and Arabidopsis root

A, BOLT is localized primarily to the nucleus in onion epidermis. B, BOLT is localized to sub-nuclear bodies in onion epidermis. C, BOLT is localized to the nucleus in Arabidopsis roots.
Many of the AP2/ERF genes in Group III respond to more than one, and often to several, abiotic and biotic stresses, so I was interested to know if BOLT responds to stresses other than the ones I tested above. Because it is impractical to test for all possible stress conditions and combinations of conditions, in addition to the above experiments, I interrogated publically available genome-wide expression data collected and curated by European Bioinformatics Institute’s (EBI) Expression Atlas and Genevestigator for conditions and genotypes in which BOLT expression changed more than two-fold (p-value <.05) (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011) (genevestigator.com). These collections each include data from thousands of microarray and RNA-seq studies in Arabidopsis and have been subject to those organizations’ in-house statistical analysis to provide a level of reliability (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011). Experiments included in these data show that BOLT was differentially expressed under stress treatments including phosphate deprivation, dehydration, salt, various types of fungi and bacteria, iron deprivation, cold stress, osmotic stress, and excess light as well as when treated with the phytohormones SA and ABA. These studies corroborate my stress treatment results suggesting that BOLT responds to both abiotic and biotic stresses (Fig. 2.3).
Figure 2.3 BOLT expression in stress-treated seedlings

Expression, measured using RT-qPCR, shows increased expression with stresses and decreases in response to SA and MeJA. Ten-day-old seedlings were treated with: A, 50µM, ABA; B 5°C temperature; C, 100mM NaCl or 300mM mannitol; D, dehydration; E, 1mM flg22 peptide; F, piercing (all leaves) with 18-guage needle; G, 400µM salicylic acid; or H, 100µM methyl jasmonate. Both β-actin and UBI10 were used as reference genes. Data represents three biological replicates and error bars show standard error of the mean.
**BOLT is differentially expressed in a range of mutant genotypes**

Transcription factors regulate gene expression as a result of elaborate signaling processes. Besides differential expression data regarding stress and growth conditions, Genevestigator and Expression Atlas (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011);(https://genevestigator.com) also have a large amount of data related to gene expression in mutant genotypes. I wanted to know what upstream genes affect *BOLT’s* expression, to possibly shed some light on a pathway, or pathways, that might regulate *BOLT*. To do this I analyzed the genotype data at the two sites and found that *BOLT* is highly differentially expressed in the *LEC1, LEC2, FUS3*, and *ABI3* group of genes that regulate embryo development, as well as in *PHYB, PFT1*, a *PIF* quadruple mutant, and *PSAD1*, which are all involved in light signaling and photosynthesis, and also a group of genes, *PAD4, SIZ1, SID2*, and *NPR1* that are involved in SA biosynthesis and regulation, and defense, and both SIZ1 and NPR1 have been shown to localize to some type of nuclear bodies (Saleh et al. 2016) (Miura et al. 2005; F. Wang & Perry 2013; Sharrock et al. 2003; Ding et al. 2014; J. B. Jin & Hasegawa 2008). However, the genotype in which *BOLT* was most significantly regulated is the *mkk1/mkk2* double mutant. In this double mutant *BOLT* was shown to be downregulated more than 10-fold. A mitogen-activated protein kinase (MAPK) pathway that includes MKK1 and MKK2 has been shown to function in abiotic and biotic stress responses (Gao et al. 2008; Kong et al. 2012; Ichimura et al. 2006; Teige et al. 2004). Is it possible then, that *BOLT* could be in a pathway with *MKK1* and *MKK2*?

*MKK1* and *MKK2* are highly homologous mitogen activated protein (MAP) kinases, that are part of a known MAPK pathway that responds to reactive oxygen
species (ROS) signaling that arises upon perception of biotic and abiotic stresses (Pitzschke et al. 2009; Kong et al. 2012; Gao et al. 2008; Qiu et al. 2008; Mészáros et al. 2006). The two MKKs are components in a MAPK signaling cascade that includes MEKK1 and MPK4 and results in downstream stress signaling targeting, among other genes, certain transcription factors such as NPR1 WRKY25, and WRKY33 all known to have roles in biotic and/or abiotic stress response (Gao et al. 2008). A second experiment reported in Genevestigator indicates that BOLT is also downregulated in MPK4 and MKS1, a substrate of MPK4 shown to interact with stress-related transcription factors, suggesting further that BOLT could act downstream of this MAPK pathway (Gao et al. 2008; Qiu et al. 2008). Thus I became interested to see if I could corroborate the evidence that BOLT is regulated by MKK1 and MKK2 and possibly place BOLT in this stress signaling pathway (Fig 2.4).

To confirm that BOLT is regulated by MKK1 and MKK2, I obtained seeds of the mkk1 and mkk2 single mutants, and the mkk1/mkk2 double mutant (kindly provided by Peter Morris, Heriot-Watt University, Scotland). The single mutant plants are similar to wild type, but the double mutant plants are severely dwarfed (Fig 2.5). I grew the three lines alongside wild-type plants, isolated RNA from the aerial parts of three-week-old plants, reverse-transcribed cDNA, and compared BOLT expression among them using RT-qPCR. BOLT expression was 94% of wild type in mkk2, 63% in mkk1, but only 8% in the double mutant, confirming the data from Genevestigator and Expression Atlas (Fig. 2.5). The fact that BOLT expression is just 8% of wild type expression in the double mutant suggests that MKK1 and MKK2 act upstream from BOLT and regulates its expression.
To further investigate the possibility that *BOLT* is acting downstream of the *MKK1/MKK2* stress response pathway, I subjected the double mutant plants to ABA, cold, and flg22 treatments, and measured *BOLT* expression using qPCR as before. The result was that *BOLT* expression increased significantly more in the wild-type plants than in *mkk1/mkk2* plants, 8.2x vs 0.5x in ABA treated plants, 7.5x vs 2.5x in cold treated plants, and 2.6x and 1.7x vs 1.6x and -0.3x in flg22 peptide treated plants (Fig 2.5). The fact that BOLT expression is less than wild type in the double mutant under stresses also suggests that MKK1 and MKK2 regulate BOLT, further supporting the possibility that BOLT is downstream of, and regulated by the MAPK cascade that includes MKK1 and MKK2. Very interestingly, BOLT has the same expression pattern as does MEKK (Fig. 2.6) (Ichimura et al. 2006). Based on all of these results, I hypothesize that BOLT responds to both biotic and abiotic stress and acts downstream of the MEKK1→MKK1/MKK2→MPK4 MAPK signaling pathway.
SA, Pathogen Cold, Salt, Dry

:RÖS: MEKK1

MKS1

MPK4

MKK1 MKK2

SA accumulation PAD4, SID2, NPR1

FUS3, ABI3 LEC1, LEC2

MKS1

BOLT

Defense responses

Abiotic stress responses

JA
Figure 2.4 Hypothesis model

Model of MEKK1 $\rightarrow$ MKK1/MKK2 $\rightarrow$ MPK4 pathway (solid lines) based on model by Miura et al describing the MAPK cascade (Miura & Tada 2014). Colored lines indicate different pathways. Red lines indicate defense response pathways, gray lines, drought response, and green lines, cold response. The dashed lines expand the published pathway to include the possibility that BOLT is in the MAPK pathway.
A  BOLT expression in mkk mutants

B  BOLT Expression in flg22-Treated mkk1/2 Mutants

C  BOLT Expression in COLD-Treated mkk1/2 Mutants

D  BOLT Expression in ABA-Treated mkk1/2 Mutants
Figure 2.5 *BOLT* expression in wild type and mkk mutants

A, *BOLT* expression in wild type compared to mkk1, mkk2, mkk1/2 mutants measured using RT-qPCR. B, C, D, *BOLT* expression in plants treated with 1µM flg22, 24 hours at 5°C, or 50µM ABA respectively. 2.5-week-old plants were used for RT-qPCR. Both β-actin and UBI10 were used as reference genes. Data represents three biological replicates and error bars show standard error of the mean.
Figure 2.6  Tissue-specific expression of *MEKK1* and *BOLT* are similar

**Additional in-silico evidence of a wide role for BOLT stress response**

In addition to *BOLT*’s expression being downregulated in the mkk1/2 double mutant, the two web tools, Genevestigator and Expression Atlas, reported 78 additional mutant genotypes in which *BOLT* expression is affected >2x (p-value <.05) (genevestigator.com) (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011). I used the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 to provide functional annotation and classification of these genes based on Gene Ontology (GO) terms (D. W. Huang, Sherman & Lempicki 2009a; D. W. Huang, Sherman & Lempicki 2009b). GO terms classify genes using the concepts of molecular function, cellular component, and biological process to describe gene function (Ashburner, et al. 2000). The most enriched clusters included genes involved broadly in transcription regulation and defense. It is not surprising that some of the genes that regulate BOLT are transcription factors. Response to hormones, defense response, and response to dehydration are corroborated by the RT-qPCR results. Reproductive structure development and response to light, we did not expect and could suggest new roles for BOLT.

I used the TAIR GO Annotations function to compare the set of genes upstream from *BOLT* to the genome as a whole, and found the upstream set to be enriched for nuclear genes and genes that respond to abiotic or biotic stimulus, protein binding, response to stress, developmental processes, transcription, DNA or RNA binding, and signal transduction (Berardini et al. 2004) (Fig. 2.7). Genes that have been well studied tend to have more GO terms assigned them than genes that have not been studied, thus genes affecting BOLT expression may have more terms assigned per gene than all genes
in the genome. This must be considered when perusing Figure 2.7, however the data is very useful in understanding BOLT’s role in stress response. This again indicates a possible role for BOLT in development as well as one in abiotic and biotic stress.

It is informative to understand what DNA binding sites are upstream of a gene. This can suggest a possible function based on the proteins known to bind the various sites. An analysis of binding-site motifs in BOLT’s promoter using the ATcisDB at the Arabidopsis Gene Regulatory Information Server (AGRIS) (Yilmaz et al. 2010; Palaniswamy et al. 2006; Davuluri et al. 2003) revealed many known binding sequences upstream from the BOLT coding region (Table 1). Further investigation determined that these motifs have been shown to reside in promoters, and bind proteins that are related to light response and photosynthesis, transition to flowering, auxin-response, dehydration, and biotic stress responses (Table 1). Binding sites for abiotic and biotic stress responses are consistent with the results that BOLT responds to those types of stresses. It has been demonstrated that light responses, photosynthesis, and flowering are regulated by environmental stresses to coordinate an appropriate response (Takeno 2016; K. C. Wada & Takeno 2014; Takeno 2012; Chaves et al. 2009; Soitamo et al. 2008; Gollan et al. 2015).

Taken together, these results suggest that BOLT is an AP2/ERF transcription factor that responds to certain biotic and abiotic stress treatments and is potentially downstream of the MEKK1 → MKK1/MKK2 → MPK4 signaling cascade.
Figure 2.7 GO annotation of potential upstream regulators of BOLT

(Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011)

genevestigator.com) compared to the whole genome categorization using Gene Ontology at TAIR (arabidopsis.org). Comparison shows nuclear genes, genes responding to biotic and abiotic stress, and genes involved in developmental processes, as well as some others, are over-represented in the genes that potentially regulate BOLT. Not all genes have GO terms assigned whereas some genes have multiple terms assigned.
Table 1 DNA-binding sites within 3-kb upstream of the BOLT translation start site according to the AtcisDB at AGRIS (Yilmaz et al. 2010)

<table>
<thead>
<tr>
<th>Binding site</th>
<th>Function</th>
<th>Binding Site Seq.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYB2 binding site motif</td>
<td>Salt and dehydration responses</td>
<td>cttaaca</td>
<td>Abe, 1997</td>
</tr>
<tr>
<td>ABRE-like binding site motif</td>
<td>Dehydration and low temperature responses</td>
<td>cacgtgga</td>
<td>Shinozaki, 2000</td>
</tr>
<tr>
<td>DRE-like promoter motif</td>
<td>Cold and dehydration responses</td>
<td>aaccgacca</td>
<td>Maruyama, 2012; Chen, 2002</td>
</tr>
<tr>
<td>ATB2 binding site motif</td>
<td>Osmotic stress response</td>
<td>acctat</td>
<td>Satoh, 2004</td>
</tr>
<tr>
<td>BoxII promoter motif</td>
<td>Responses to light, pathogen, salt</td>
<td>ggttaa</td>
<td>Le Gourrierec, 1999</td>
</tr>
<tr>
<td>G-box promoter motif</td>
<td>Photosynthesis and stress response</td>
<td>cacgtg</td>
<td>Vandepoel, 2009; Menkens, 1994; Figueroa, 2012</td>
</tr>
<tr>
<td>Ibox promoter motif</td>
<td>Photosynthesis and stress response</td>
<td>gtaag</td>
<td>Vandepoel, 2009; Giuliano, 1988</td>
</tr>
<tr>
<td>GATA promoter motif</td>
<td>Light responsive transcription</td>
<td>tgtag</td>
<td>Teakle, 2002</td>
</tr>
<tr>
<td>T-box promoter motif</td>
<td>Light-activated transcription</td>
<td>accttg</td>
<td>Chan, 2001</td>
</tr>
<tr>
<td>SORLREP3 promoter motif</td>
<td>Light-regulated gene expression</td>
<td>tgtatatat</td>
<td>Hudson, 2003</td>
</tr>
<tr>
<td>SORLIP2 promoter motif</td>
<td>Light-regulated gene expression</td>
<td>gggcc</td>
<td>Hudson, 2003</td>
</tr>
<tr>
<td>MYB4 binding site motif</td>
<td>UV-B response</td>
<td>aacaaac</td>
<td>Zhao, 2007; Chen, 2002</td>
</tr>
<tr>
<td>Bellringer binding site motif</td>
<td>Flower development</td>
<td>aaattaa</td>
<td>Bao, 2004</td>
</tr>
<tr>
<td>ARF1 binding site motif</td>
<td>Auxin response</td>
<td>tgtctc</td>
<td>Boer, 2014; Ulmasov, 1999</td>
</tr>
<tr>
<td>ATHB2 binding site motif</td>
<td>Auxin response, circadian control, embryo dev.</td>
<td>taataatta</td>
<td>Kunihiro, 2011; Turchi, 2013; Sessa, 1993</td>
</tr>
<tr>
<td>ARF binding site motif</td>
<td>Auxin response</td>
<td>tgtctc</td>
<td>Boer, 2014</td>
</tr>
<tr>
<td>W-box promoter motif</td>
<td>Defense response</td>
<td>tggacc</td>
<td>Ciolkowski, 2008; Yu, 2001</td>
</tr>
<tr>
<td>CCA1 binding site motif</td>
<td>Circadian rhythm</td>
<td>aaaaatct</td>
<td>Andronis, 2008</td>
</tr>
<tr>
<td>DPBF1&amp;2 binding site motif</td>
<td>ABA response and embryo-specification</td>
<td>acacgtg</td>
<td>Kim, 1997</td>
</tr>
<tr>
<td>RAV1-A binding site motif</td>
<td>Germination and early seedling development</td>
<td>caaca</td>
<td>Feng, 2014; Kagaya, 1999</td>
</tr>
<tr>
<td>RAV1-B binding site motif</td>
<td>Germination and early seedling development</td>
<td>cacctg</td>
<td>Feng, 2014</td>
</tr>
<tr>
<td>LFY consensus binding site motif</td>
<td>Transition to flowering and biotic stimulus response</td>
<td>ccacctg</td>
<td>Winter, 2011</td>
</tr>
<tr>
<td>L1-box promoter motif</td>
<td>Tissue specific expression, L1 layer</td>
<td>taaatgta</td>
<td>Abe, 2001</td>
</tr>
<tr>
<td>RY-repeat promoter motif</td>
<td>FUS3 and ABI3 binding site, embryogenesis</td>
<td>catgcatg</td>
<td>Reidt, 2000</td>
</tr>
</tbody>
</table>
Discussion

In Chapter 1, I presented evidence that BOLT is a potential AP2/ERF transcription factor, and that many related transcription factors respond to abiotic and biotic stresses. In this chapter I ascertained BOLT’s expression pattern and sub-cellular localization, as well as determined that its expression level changes when subjected to ABA, SA, MeJA, cold, dehydration, salt mannitol, flg22, and wounding treatments. My experiments show an interesting expression pattern, especially the sub-cellular localization, and provide evidence that BOLT is potentially downstream of the MEKK1→MKK1/MKK2→MPK4 signaling cascade.

BOLT is expressed widely in the plant

According to the results of the GUS staining experiments, BOLT is expressed most in flowers, embryos, and young leaves. Older leaves and roots show expression mostly in the vasculature. The RT-qPCR results show more expression in the flowers and leaves than in the roots. These results, interesting on their own are even more so as they agree with the spatial expression patterns of MEKK1 and MPK4 reported by Ichimura et al (Fig. 2.7) (Ichimura et al. 2006). Both MEKK1 and MPK4 have strong expression in vascular tissue and MEKK1 is more strongly expressed in emerging true leaves when compared to mature leaves, both similar to BOLT (Fig 2.7) (Ichimura et al. 2006). My RT-qPCR and GUS staining results indicating higher expression in flowers and leaves than in roots, as well as dark staining in the embryo are also corroborated in a transcription profiling experiment designed to show organ-specific expression (J. Liu et al. 2012). In that microarray data, BOLT expression was more highly expressed in
flowers and seeds and somewhat less so in leaves. In roots the expression level was
below the cut-off for the experiment (Petryszak et al. 2013; Petryszak et al. 2016;
Kapushesky et al. 2011).

For the GUS staining experiments, I used 1666 base pairs of BOLT’s promoter to
make the GUS lines. It is possible that this promoter is not of sufficient size to elucidate
BOLT’s entire expression pattern, however since the GUS staining and qPCR
experiments both show expression in flowers, leaves, and roots, I believe the information
is meaningful.

**Some transcription factors localize to nuclear bodies**

A very interesting result is the sub-nuclear localization of the YFP signal in the
transgenic lines in transiently transformed onion epidermis. In the onion epidermal cells,
BOLT did not localize evenly in the nucleus, but rather the signal was punctate (Fig. 2.2).
Nuclear bodies are common and dynamic structures found in eukaryotic nuclei. Plant
nuclear bodies are membrane-less sub-nuclear organelles of various types such as the
nucleolus, Cajal Bodies, nuclear speckles, dicing bodies, and photobodies ((Petrovska et
al. 2015). These structures vary in size, shape, and number, and in some cases their
dynamics have been linked to environmental stresses (Reddy et al. 2012; Geilen &
Böhmer 2015).

There are some examples of proteins that are shown to localize to nuclear bodies,
but how they are formed and maintained is not clear (Mao et al. 2011). An important
regulatory mechanism for managing transcription factors is cellular compartmentalization
(Rim et al. 2011). The most pertinent example is that transcription regulators SIZ1 and
NPR1 have both been shown to localize to nuclear bodies (Miura et al. 2005; Saleh et al. 2016). According to publicly available expression data, *BOLT* is differentially expressed in npr1, siz1 and pad4 mutants (Kapushesky et al. 2011; Petryszak et al. 2013; Petryszak et al. 2016). SIZ1 is required for PAD4-mediated defense signaling and siz1 mutants accumulate SA. NPR1 is a transcriptional regulator of defense response (Saleh et al. 2016). Both genes also have roles in flowering time regulation (J. B. Jin & Hasegawa 2008; G.-F. Wang et al. 2011). These data suggest that BOLT could interact with NPR1 or SIZ1 in nuclear bodies possibly in a role that affects flowering time through defense response.

To determine if BOLT associates or interacts with these proteins, tagged SIZ2 or NPR1 could be co-expressed with BOLT in onion epidermal cells. An overlapping signal would indicate co-localization. Interaction could then be determined using Bimolecular Fluorescence Complementation (BiFC) or Co-Immunoprecipitation (Co-IP).

It has also been shown that WRKY18 and WRKY40, both pathogen-induced transcription factors, co-localize with PIF3, PIF4, and PHYB to Phytochrome B-containing nuclear bodies (PNBs). When treated with ABA, WRKY40 moves from the PNBs to the nucleoplasm (Geilen & Böhmer 2015). It has been suggested that light responses are mediated in photobodies by transcriptional regulation. This is supported by the fact that many of the known photobody constituents are either photoreceptors or transcription factors (Jiao et al. 2007; Van Buskirk et al. 2012). *BOLT* is regulated in a phyb mutant per an experiment in the Genevestigator database (genevestigator.com). This suggests that BOLT could have a role in the response to light possibly integrating environmental and development cues.
In addition, it is known that MKS1 forms a complex with MPK4 and WRKY33 in the nucleus of unstressed cells. Upon treatment with \textit{P. syringae} or flg22, MPK4 phosphorylates MKS1 and WRKY33 is released from the complex (S. Liu et al. 2015). This is especially interesting since \textit{BOLT} expression may be regulated by MKS1 (genevestigator.com). Other examples of stress-related transcription factors localizing to nuclear bodies are, ERF4, a negative regulator of JA-responsive defense gene expression, and AFP, a negative regulator of ABA signaling, both of which have been shown to localize to nuclear bodies, potentially controlling the activity of the protein (Z. Yang et al. 2005; Lopez-Molina et al. 2003). Interestingly, in these three examples, each of the transcription factors is considered a negative regulator of transcription.

In the YFP localization experiments, my aim was to determine where BOLT localized under normal growing conditions. The fact that one of the experiments was done in onion epidermal cells may not necessarily show this because BOLT could localize there due to stress, or dark, or light, all conditions the onion cells experienced until it was viewed under the microscope. The other localization experiment, however, was done in transgenic \textit{Arabidopsis} under normal growing conditions and in these cases the YFP signal also localized to the nucleus suggesting this is the actual localization under normal conditions. That said, it would be interesting to do additional experiments in both onion and \textit{Arabidopsis} comparing stressed and unstressed conditions as well as different light conditions to see if the different environments have an impact on protein localization.

The BOLT::YPF constructs were created using a vector containing a CaMV35S promoter which causes overexpression of \textit{BOLT}. Ideally, a native promoter would have
been used in the localization of the protein, however since it is evident from the microscopy results that BOLT localizes solely to the nucleus, and the expression pattern as evidenced by the GUS staining agrees, it appears that in this case using the overexpressor did not produce misleading localization results. If the experiments discussed in the previous paragraph were undertaken, generating constructs using a native promoter would be a preferable first step.

**BOLT may negatively regulate defense**

*BOLT* is downregulated more than ten times in the *mkk1/mkk2* double mutant and publically available microarray results propose that it is also downregulated in *mkp4*. The MAPK pathway has been shown to negatively regulate plant immunity (Kong et al. 2012) If *BOLT* is positively regulated by the MAPK pathway, this suggests BOLT’s role could be one of negative regulation in response to stress (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011).

It is interesting that in the stress treatment experiments *BOLT* expression was upregulated within 15 minutes of treatment with flg22, but that the expression dropped off by shortly afterwards (Fig 2.3), but that *BOLT*’s response to cold was very different. Expression in response to cold increased somewhat within 90 minutes, but then significantly by 24 hours. This could suggest that although the responses may be mediated by the same MAPK pathway, the mechanism is different for the two types of stress possibly indicating that different genes are involved (Fig2.3). This agrees with results showing separate pathways through MKK1 and MKK2 to MPK4 for different stresses and is supported by recent studies suggesting that various concurrent stresses
result in responses different from the responses to either stress and are as if the plant is responding to a new state of stress (Miura & Tada 2014; Aarti Gupta 2016; Rasmussen et al. 2013).

Further support for the conjecture that BOLT could be a negative regulator of stress responses is that PAD4, SID2, and NPR1 all downregulate BOLT according to data at genevestigator and Expression Atlas (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011). These three genes are shown to have a role in biotic stress responses through the biosynthesis and accumulation of, and response to salicylic acid.

PAD4 is an essential component of defense and participates in a defense amplification loop that responds to salicylic acid and ROS, while MPK4 negatively regulates SA accumulation (Petersen et al. 2000; Feys et al. 2001; Feys et al. 2005; Song et al. 2004; Jirage et al. 1999). In the presence of SA NPR1 translocates to the nucleus to effect gene expression of downstream defense genes (Fan & Dong 2002; C. Johnson et al. 2003). SID2 is required for SA production upon pathogen infection and is negatively regulated by MEKK1 (Ichimura et al. 2006). There is apparent antagonism between these genes involved in the biosynthesis and accumulation of SA and MPK4 which negatively regulate the accumulation of SA and it is interesting that BOLT is downregulated by the former and upregulated by the latter. This suggests an SA dependent role for BOLT and the possibility that BOLT mediates SA signaling.

According to RNA-seq and microarray experiments at Genevestigator and EBI (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011) LEC1, FUS3, ABI3, and LEC2, embryogenesis regulators, positively regulate BOLT. The data show BOLT downregulated in the mature green seeds of knockout mutants and upregulated in an
LEC1 overexpressor (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011). These four genes are known to control embryo and seed development, and have more recently been shown to be involved in the transition from embryo to seedling development (Harada 2001; Y. Yamamoto et al. 2010; F. Wang & Perry 2013). This is interesting because in the GUS staining experiments, embryos stained very dark blue. In addition, there are two RY-repeat promoter motifs in BOLT’s promoter. Reidt, et al demonstrated that the RY motif is a target for FUS3 and ABI3 further supporting the regulation of BOLT by these development genes (Reidt et al. 2000). This is interesting data suggesting either an additional role for BOLT in development, or an additional role for the development genes, FUS3 and ABI3 in stress response.

**Materials and Methods**

**Plant growth**

Seeds were surface sterilized in 25% bleach with 0.01% Triton-X, a surfactant, for 10 minutes then washed six times in sterile, nano-pure water. The sterilized seeds were plated on 1/2MS medium (pH 5.8) solidified with 0.8% phytoagar, placed in the dark at 4°C for four days for stratification, then transferred to growth chambers. Plants (Arabidopsis thaliana, ecotype Columbia-0) were grown at 22°C under 100uM/m²/s continuous light. Plates containing YPF lines were grown vertically enable root imaging. For soil growth, pots were stratified and grown under the above conditions.

**Generation of transgenic plants**
The expression pattern was studied using the β-glucuronidase (GUS) reporter gene system. A DNA fragment from the translation start site and including 1666-bp of the promote region was cloned from the F6F3 BAC purchased from ABRC using the following primers to add Gateway attB sequences and amplified by PCR.

Fwd: 5’ggggACAAGTTTGTACAAAAAAGCAGGCTTCAGTTACTTACTGTGGTTTTTTAAAAA CG 3’

Rev: 5’ggggACCACTTTGTACAAGAAAGCTGGGGTTTTAAAGAGTTTGTATGTGTAGTC 3’

Using the Gateway cloning system, the PCR product was cloned into pDONR-Zeo, and the resulting entry clone was recombined with pMDC164 to produce the expression vector (Curtis & Grossniklaus 2003).

Sub-cellular localization was studied using a yellow fluorescent protein (YFP) reporter. The BOLT CDS was cloned from ABRC DNA stock U84819, using the following primers to add Gateway attB sites and amplified by PCR.

Fwd: 5’ggggACAAGTTTGTACAAAAAAGCAGGCTTCATGTCACCACAGAATGAAGCTATCATC 3’

Rev: 5’ggggACCACTTTGTACAAGAAAGCTGGGGTTTCACAGACCCATGAACTGTA TACTG 3’
Using the Gateway cloning system, the PCR product was cloned into pDONR-Zeo and the resulting entry clone was recombined with pEarleyGate101 (C-terminal YFP) or pEarleyGate104 (N-terminal YFP) to produce the expression vectors (Earley et al. 2006).

Arabidopsis Col-0 plants were transformed per the Clough and Bent protocol (Clough & Bent 1998). Homozygous transgenic lines were selected using Basta. T1 plants were sprayed and the seeds of the heterozygous survivors (T2) were again selected for. T3 seeds were planted and sprayed with Basta and the homozygous lines (100% survival) were used in further testing. The lines were also confirmed using microscopy to visualize the YFP reporter.

**Histochemical β-glucuronidase (GUS) reporter gene expression**

Transgenic seedlings were treated according to the Franks lab protocol, North Carolina State University. Whole seedlings were placed in 90% acetone, a vacuum applied for 15 minutes, then 30 minutes on ice. Acetone was removed and staining buffer without X-Gluc added. Vacuum applied for 15 minutes. The buffer was removed and staining buffer containing X-Gluc was added and put in vacuum for 15 minutes. The samples were incubated in the dark at 37°C overnight (~14 hours). Staining buffer was removed and samples were incubated at room temperature for 30 minutes each in ethanol at 20%, 35%, 50%, and 70%. Staining buffer includes: 50mM NaPO4 buffer, pH7.2, 0.2% Triton X, 2mM Potassium Ferrocyanide, 2mM Potassium Ferricyanide, and 2mM X-Gluc (5-bromo-4-chloro-3-indoly1 β-D-glucuronide cyclohexamine salt).
Onion epidermis bombardment

Onion bombardment was performed as described by Hollender and Liu (2010). Onion pieces were incubated for 16 hours in the dark.

Treatments with hormones and stresses

Ten-day-old seedlings grown as described above were treated by spraying with 50µM ABA, 500µM SA, or 1mM flg22. Control plants were sprayed similarly but without the treatment chemical. Cold treatment was applied by placing the plants at 4°C under the same lighting conditions. For dehydration treatment, plates were opened in a hood under light. Wounding treatment consisted of puncturing each leaf with an 18-gauge needle.

Quantitative RT-PCR

RNA was isolated from 100mg of seedlings using the Spectrum Plant Total RNA Kit from Sigma (STRN250-1KT). Samples were treated with DNase (On-Column DNase I Digestion Set – DNASE70-1SET). RNA concentrations and contamination were measured using the NanoDrop 2000c, and RNA integrity was assessed gel electrophoresis. 1µg RNA was reverse transcribed (20ul reaction) using the iScript Advanced cDNA Synthesis Kit (BioRad catalog #172-5038). 1µl of the resulting cDNA was used in 10µl qPCR reactions with iTaq Universal SYBR Green Supermix (BioRad catalog #172-5121) and 500ng each primer. Samples processed using a CFX96 Real-Time PCR Detection System (BioRad). Results were analyzed using CFX Manager software v.3 (BioRad).
qRT-PCR Primers were designed using NCBI’s Primer-BLAST ((Ye et al. 2012).

BOLT

Fwd: 5’-GGCAGGAATAGACGACGGAG-3’
Rev: 5’-AACACTCACAGACACGCCAT-3’

MKK1

Fwd: 5’-TCTGACCTTGTCTCTGTGC-3’
Rev: 5’-TGGATTGCTCAAGAGGAGG-3’

MKK2

Fwd: 5’-TCCTGAGGAAAGGTTTTGTTC-3’
Rev: 5’-TGGAGACAGGACTTCAGGCT-3’

PP2A (control)

Fwd: 5’-GGGTGATTATGTTGATCGAGGT-3’
Rev: 5’-TGACGGCTTTTCATGATCCCCT-3’

UBI10 (control)

Fwd: 5’-AGAAGGAATCCACCCTCCAC-3’
Rev: 5’-GCAAGAGTTCTGCCATCCTC-3’

Protocol:

1: 95.0°C for 3:00
2: 95.0°C for 0:10
3: 60.0°C for 0:30

Plate Read

4: GOTO 2, 39 more times
5: 95.0°C for 0:10

6: Melt Curve 65.0°C to 95.0°C: Increment 0.5°C 0:05

Plate Read

Microscopy

A Zeiss LSM 710 Confocal Microscope was used to observe and photograph onion epidermis. A Zeiss AxioObserver inverted fluorescence microscope was used to observe and photograph Arabidopsis roots.
Chapter 3: *BOLT* overexpression results in early bolting and altered response to light intensity; knockdown shows dehydration tolerance

Introduction

I named BOLT for the early bolting phenotype exhibited by the overexpressing lines which are reported in this chapter. In Chapter 2 I presented evidence that BOLT, a putative transcription factor, is widely expressed in the plant and responds to biotic and abiotic stress treatments. I was then interested to see what effect knocking-out or overexpressing BOLT would have on the plant. Would the loss or overproduction of the gene cause an identifiable phenotype and help to elucidate BOLT’s role? To understand the role of BOLT through analysis of phenotypes, I used both knockout and overexpression strategies.

Two common methods for studying the roles of transcription factors, as well as other genes, are investigating the phenotypes in either knockout or in overexpressing lines. Growing transgenic plants alongside the wild type can highlight phenotypic differences caused by the altered expression of the gene in question. These phenotypes can suggest possible functions for the transcription factor.

Loss-of-function mutants (knockouts or knockdowns) have been used successfully to suggest gene function in many species from bacteria, to yeast, *C. elegans*, and *Arabidopsis* (Giaever et al. 2002; Kamath et al. 2003; Thorneycroft et al. 2001). An enormous amount of information has been generated using knockout mutants. It has been noted though, that for a large percentage of knockout mutants, no phenotype is observed (Kamath et al. 2003). It was hypothesized that the reason for this is functional
redundancy due to gene duplication (J. Z. Zhang 2003). In *Arabidopsis*, this seems to be the case as considerable gene duplication has been shown (Vision et al. 2000; Simillion & Vandepoele 2002). Functional redundancy is particularly of concern in the study of transcription factors because they are generally members of large gene families that often include closely related genes (Riechmann et al. 2000).

Overexpression is an alternative strategy to knockout/knockdown analysis that is less affected by functional redundancy but can also result in mutant phenotypes as gene dosage is important for normal gene function (Prelich 2012; J. Z. Zhang 2003; Spadafora et al. 2012). Balanced gene expression is important and even small changes in copy number or concentration can cause mutant phenotypes (Prelich 2012). Overexpressing lines have been used to show a phenotype in *Arabidopsis* when knockouts did not (J.-X. Liu et al. 2009). In some instances, even when a knockout mutant has an informative phenotype, overexpression has generated different and unexpected phenotypes suggesting additional roles for some transcription factors (T. Wada et al. 2002; Schellmann et al. 2002).

Overexpressors usually, but not always generate gain-of-function phenotypes. These can either result in hypermorphs, which display the result of an increase in otherwise normal gene function, or in neomorphs, which exhibit a different phenotype from the endogenous gene, possibly the result of incorrect tissue or developmental stage expression, or off-target binding due to overabundance. (Prelich 2012; J. Z. Zhang 2003). In considering any phenotypes observed in the case of overexpressors, we have to keep these possibilities in mind.
Results

Generation of transgenic lines

Initially, since I was interested to know if I could observe a different phenotype in plants lacking BOLT. I analyzed several existing Arabidopsis T-DNA lines, GABI_914CO4, purchased from Nottingham Arabidopsis Stock Centre (NASC), and Salk_044673.42.65 and SM-3_34537 both purchased from ABRC. The GABI-Kat T-DNA line had been purchased by a previous postdoc in the Kwak lab. In investigating the line at the Salk website (signal.salk.edu), I found the line had additional T-DNAs in two other genes, so I discontinued using that line (signal.salk.edu). Salk_046673.42.65 has only one hit per the Salk website, however, results of my PCR to locate the T-DNA showed it to be in the 3’ UTR and RT-PCR showed gene expression. The third line, SM-3_34537, did not result in a full length mRNA, however did result in expression using two qPCR primer sets, which made it unclear if the T-DNA would knock down BOLT’s function (Appendix A).

Since the available T-DNA lines did not yield a knockdown of BOLT, I decided to use RNA interference (RNAi) and generated plants expressing artificial microRNAs against BOLT (RNAi lines). To accomplish the experiments comparing the phenotypes of wild-type plants with plants deficient in BOLT, I transformed plants with a plasmid encoding an artificial microRNA. I identified fifteen homozygous lines, and subsequently chose the first four lines tested by qPCR that had very low expression, to use in subsequent experiments (Fig. 3.1). In the four lines, I found that BOLT expression was knocked down to less than 10% of wild-type expression (Fig 3.1). I initially grew the
RNAi plants next to the wild type in normal growing conditions (22°C and 115µM/m²/sec 16-hour light), but observed little difference between the lines in leaf size, shape, or color, rosette size, or flowering time. Figure 3.1 includes illustrative photographs of RNAi plants compared to wild type.

Transcription regulation is often redundant, so possibly a knockdown phenotype was masked by another gene causing the lack of observable phenotype in the RNAi lines. The plants were grown under normal conditions, and since we hypothesize that BOLT may be required under stress conditions, this too could be the reason we did not see a phenotype.

Overexpressing lines have often been used successfully in characterizing transcription factors. With this in mind, I made transgenic lines that included a 35S::BOLT::YFP. I selected several dozen independent homozygous overexpressing lines, examined seedling roots under a fluorescent dissecting microscope for YFP signal and tested the expression of eight lines that had a signal. Most of the two dozen lines had a visible YFP signal. I chose six lines with different levels of expression, in which the plants appeared to grow well to use in further experiments (Fig. 3.2).

**Overexpressing plants bolt earlier than wild type**

I planted the six overexpressing lines along with wild type, and grew them under the same normal growing conditions (115µM/m²/sec 16-hour light at 22°C). By two weeks, the transgenic plants looked similar to wild type. I watered the two-week-old plants and left them until the next watering. When I returned to the plants a week later, the overexpressing plants had all bolted while none of the wild-type plants even had a bud. This phenotype is the reason I call the gene, At1G01250, BOLT.
BOLT Expression in RNAi lines

Normalized expression

Line

wt RNAi-25 RNAi-19 RNAi-14 RNAi-8 RNAi-7

wt RNAi

wt RNAi

wt RNAi
Figure 3.1  *BOLT* expression in RNAi lines, and RNAi plants compared to wild type

Plants grown under 115µM/m²s light and 16-hour photoperiod. A, *BOLT* expression measured using RT-qPCR in five independent RNAi lines compared to wild type. Photographs, 2.5-week-old plants, wild type (top left) and RNAi-25 (top right). 3.5-week-old plants, wild type (bottom left), and RNAi-25 (bottom right). In RT-qPCR, both β-actin and UBI10 were used as reference genes. Data represents three biological replicates and error bars show standard error of the mean.
Figure 3.2  *BOLT* expression in overexpressing lines compared to wild type

Plants were grown under 115µM/m²s light and 16-hour photoperiod. Expression was measured using RT-qPCR. β-actin and UBI10 were used as reference genes. Data represents three biological replicates and error bars show standard error of the mean.
This early flowering was very surprising since at first there appeared to be few differences between the transgenic lines and the wild type. The RNAi lines had not yet bolted. They subsequently bolted at about the same time as the wild type. I considered what, aside from the overexpression, could have caused the early flowering phenotype. The plants were crowded in 3-inch pots, or they may have been too dry. The humidity is uncontrollable in the chamber which affects plant growth. The wild-type plants were under the same conditions as the overexpressors, but to investigate whether one of these other possibilities was contributing to the difference in flowering time, I planted 16 plants per line, and distributed the lines throughout the trays and the trays throughout the chamber to account for any small differences in light, water, or temperature that may have affected the earlier results. Again, all of the overexpressing lines bolted well before the wild-type.

This suggested that the early flowering was not due to the stress of crowding, dehydration, or small differences in conditions at different points in the chamber. I then wanted to do a larger experiment and to collect data on the difference in flowering time, and other observable differences, between the overexpressing lines, the RNAi lines, and the wild type, and quantify the differences I had seen among the lines. Up to this point, all of the plants had been grown in the same chamber under the same conditions, so I grew the same lines under the following light conditions: 24-, 16-, or 12-hour normal light (115µM/m²/sec) or 24-hour low light (50µM/m²/sec). I did this experiment twice, growing a total of sixteen plants per line, per condition. All of the chambers were kept at 22°C and approximately 60% humidity, although the humidity could not be controlled in the chambers. The aim in doing this was to make sure the phenotype wasn’t connected to
the conditions in the one particular chamber and to determine if the various light conditions would cause phenotypes.

**Early flowering in BOLT overexpression lines is photoperiod-independent**

One of the striking phenotypes of the overexpressing lines is that the plants consistently bolt earlier than wild type, up to two weeks earlier under some conditions. In all experiments I considered plants to have bolted when the stem measured 1mm. For the plants grown in normal light (115µM/m²/sec), the wild-type plants bolted at an average of 20-days-old in continuous light, 27.8 days in 16-hour light, and 50 days in 12-hour light, while the overexpressing lines flowered earlier, at an average of 17.2, 21.6, and 39.6 days respectively (Fig. 3.3). The RNAi lines bolted slightly earlier than wild type (Fig. 3.3). The overexpressing plants also had fewer rosette leaves at the time of bolting. The number of leaves at bolting for wild type was 11.5 in continuous light, 13.25 in 16-hour, and 22 in 12-hour light. The overexpressing lines had fewer leaves: 7.2, 7.9, and 15 respectively (Fig. 3.3). This could be due, at least in part, to the younger age at which the overexpressors bolted. In all three photoperiods the RNAi lines flowered slightly earlier than wild type (Fig 3.3). The evidence that the overexpressing lines all bolted early despite the hours of light suggests BOLT’s role in early flowering is photoperiod-independent.
Figure 3.3 Flowering time comparison

Overexpressing lines (OE), RNAi lines and wild type. Plants grown under 115μM/m²'s light and varying photoperiods. A, age, in days, at which each line bolted. B, number of leaves on each plant when it bolted. A plant is considered to have bolted when it has a 1mm stem. Error bars show standard error of the mean.
Figure 3.4 Overexpression lines have larger rosette size under 12-hour photoperiod.

All plants grown under 115µM/m²s light. Top, 12-hour photoperiod. Middle, 16-hour photoperiod. Bottom, 24-hour photoperiod.
A second interesting phenotype I found was that the photoperiod does have an effect on the relative size of the rosettes in the overexpressing lines compared to wild type under normal light levels. Thus, certain phenotypic aspects of overexpressing BOLT seems to be dependent on the photoperiod. Under continuous light and the 16-hour photoperiod, the overexpressing plants and wild-type were similarly sized, but under a 12-hour (short day) photoperiod, most of overexpressing lines were markedly larger than wild type (Fig. 3.4). This result suggests that BOLT has a role in plant growth that is dependent on the photoperiod, introducing the possibility that BOLT affects genes in growth pathways.

**Low light intensity leads to small/weak plants in overexpressing lines and larger plants in RNAi lines**

I also compared plants grown under continuous normal light intensity (115µM/m²/sec) to plants grown in continuous low light (50µM/m²/sec). The most interesting low-light phenotype, is that the plants grown under low light were much smaller than wild-type with spindly stems, while the RNAi lines were larger than wild type (Figs 3.5). The overexpressing plants, even before bolting, could not remain upright (Fig. 3.5). These phenotypes were only observed under low intensity light suggesting a distinct role for BOLT under low light intensity, possibly separate from the one that results in early flowering.

Under normal light intensity the average overexpressing plant bolted 17.2 days after cotyledons appeared, approximately three days earlier than wild type. Under low light, however, the difference was greater. Wild-type plants bolted at an average of 33
days, but the overexpressors bolted at 21.5 days (Fig 3.6). The RNAi plants bolted earlier than wild type as well, but not as early as overexpressors. In both light intensities, the overexpressing plants had fewer leaves when they began to bolt than did the wild type, which is a typical early flowering phenotype, and so did the RNAi lines, but again the difference between them and wild type was much less. Under normal light, wild-type plants had an average of 11.5 leaves, and under low light, 13.75. Overexpressing plants had an average of 7.2 leaves under normal light, and 6.5 under low light (Fig 3.6). While the wild-type plants had more leaves at bolting under low light than under normal light intensity, the overexpressors did not.

These results taken together suggest a possible dual role for BOLT. It appears that the early flowering in the overexpressors is not related to the photoperiod or to light intensity since it happens in all the light conditions. The small/weak overexpressing phenotype however, clearly is related to the light intensity. Could BOLT have separate roles, one depending on light intensity? We hypothesize a role for BOLT in stress response thus early flowering could be an escape mechanism from stress that threatens the current generation. In the second instance, it seems reasonable to propose that BOLT downregulates a pathway that is required in low light but not in normal light.
Figure 3.5 Overexpressing plants under low light

In low light, RNAi plants are larger and overexpressing plants are smaller than wild type. All plants grown in low (50µM/m²s) continuous light. Top, 3-week-old plants. Bottom, 4.5-week-old plants.
Figure 3.6  Flowering time and number of leaves at bolting

Plants grown under normal (115μM/m²s) or low (50μM/m²s) light intensity. A, average age in days at which plants bolted. B, average number of rosette leaves present at the time of bolting. n=8. Plant considered to have bolted if stem was 1mm.
Other phenotypes

In addition to the early flowering and light intensity phenotypes discussed above, there were differences in the leaf morphology of the overexpressing plants compared to wild type and the RNAi lines. In all conditions, the leaves of the overexpressors are flatter than wild type and the RNAi lines (Fig 3.7). The seeds of the overexpressing plants were somewhat smaller and lighter colored than wild type seeds while the RNAi lines seeds were slightly larger (Fig.3.8). I did not collect statistics on either of these phenotype, only noticing them during the course of my other experiments. The flat-leaf phenotype was irrespective of photoperiod or light intensity, but did seem more pronounced in shorter days. In low light, the phenotype was less distinct because the wild type leaves were flatter than under stronger light.

Drought tolerance in RNAi lines

Since BOLT expression responds to stress, I thought that possibly the RNAi lines might show an opposite phenotype to the overexpressing lines, such as flowering later than wild type, in response to being stressed. To test this, I subjected plants beginning at 1.5 weeks of age to wounding, cold temperature, and dehydration stresses over the course of four weeks. The wounded plants bolted at the same time as the wild-type and were no different in physical appearance. The plants subjected to cold stress also were indistinguishable from wild-type, and none of the wt or RNAi lines bolted over the course of four weeks, most likely due to the cold temperature. The dehydrated plants also flowered at about the same time as wild type.
The dehydration experiment consisted of watering plants well for 1.5 weeks, suspending watering for 3 weeks, and watering the plants again at that point. At the end of 2.5 weeks, the RNAi plants were much greener than wild type (Fig. 3.9). The plants also recovered after re-watering whereas the wild type did not (Fig. 3.9). These results suggest BOLT could have an inhibitory role with respect to drought response since dehydration increases $BOLT$ expression, but the RNAi lines exhibit drought tolerance.
Figure 3.7 Overexpressing plants have flatter leaves than wild type or RNAi plants
Left, 14-hour photoperiod 115µM/m²'s light. Right, 16-hour photoperiod, 115µM/m²'s light.
Figure 3.8 Relative seed size in BOLT overexpressing and RNAi lines

n=50 in each of two experiments. Images were collected with a light microscope and ImageJ was used to measure the area. Error bars show standard error of the mean.
Figure 3.9 RNAi lines are more tolerant to dehydration than wild type.

Top, no water for 18 days. Bottom, rewatered after 22 days. Photograph is on day 25.
Discussion

**RNAi lines suggest BOLT inhibits drought tolerance**

My experiments growing the RNAi knockdown resulted in no observable phenotypic differences between the RNAi lines and the wild type under normal growing conditions. As with many genes in *Arabidopsis*, there could be functional redundancy with one or more genes compensating for the loss of *BOLT*. Extensive duplication of the genome over evolutionary time is apparent and there are large numbers of transcription factors with related binding domains and conserved motifs (Simillion & Vandepoele 2002; Vision et al. 2000; Riechmann et al. 2000). It would be a mistake to think that plants would have evolved without sufficient redundant systems in place to ensure their continued survival. If this is the case with *BOLT*, comparing wild-type to RNAi lines will not result in a loss of function phenotype. A possible solution using knockout lines would be to knockout the two genes closest to *BOLT* in Nakano’s phylogeny, and engineer a triple mutant (Fig 1.2). Comparing wild type to the single and triple mutants might produce an informative phenotype if the redundancy is the result of one of those genes.

The drought tolerant result in the RNAi line suggests that we can see a phenotype in the loss-of-function lines (Fig. 3.9). The most probable reason the RNAi lines do not show a phenotype under stress conditions is that since *BOLT* is induced by stress (Fig. 2.3), it may not be required for success of the plant under normal growing conditions. I attempted to address this possibility by stressing RNAi lines and comparing them to wild type. The drought stress experiment in which watering was stopped for 3 weeks resulted in the RNAi plants displaying a greater tolerance to dehydration than the wild type. This
is significant because it suggests that BOLT may act under stress conditions. It is also interesting because instead of showing that BOLT is necessary under drought conditions, it shows that BOLT is inhibitory in that situation. The plants lacking BOLT were drought tolerant, not the wild type. This suggest a role for BOLT in the inhibition of drought stress response which could indicate that type of role in other types of stress as well. This is useful information that can benefit experimental design going forward in the investigation into a role for BOLT in Arabidopsis.

Another possible reason for the lack of phenotype in the RNAi lines under normal conditions could be that very small differences between the two genotypes might not be detectable, or could be masked by inconsistent growing conditions, such as different positions in the growth chamber or trays, which can cause slightly different light, temperature, or humidity conditions for each plant. Humidity in the different chambers, or in one chamber over time varies considerably depending on the number of plants in the chamber and whether they are dry, well-watered, or over watered. This could affect experimental results. I grew many, many plants over the course of these experiments, and in all cases the RNAi lines appeared similar to wild type, but if more perfectly controllable conditions were available they might be used to show differences between the line that I was not able to observe.

When using a knockdown line rather than a knockout line, there is the additional the possibility that there may be enough of the protein produced to disguise a mutant phenotype. I do not think this is the case with these BOLT RNAi lines however as the expression of all of the lines used in experiments is less than 10% of the wild type.
Early flowering may be a stress response

Observing such a striking early flowering phenotype in the BOLT overexpressing plants under all light conditions tested indicates that the gene may play a role in transition to flowering. In Chapter 2 we saw that BOLT responds to a range of stress treatments. It is possible that the early flowering seen in the BOLT overexpressing lines is an escape mechanism to avoid stress and protect reproduction. Evidence that stress induces the transition to flowering is increasing in the literature (Takeno 2016; K. C. Wada & Takeno 2014; Takeno 2012; Blanvillain et al. 2011; Yaish et al. 2011; Riboni et al. 2014; Kazan & Lyons 2015; Kolár & Senková 2008; Riboni et al. 2013).

Drought stress promotes flowering under long day conditions in Arabidopsis (Riboni et al. 2013), and my results that BOLT expression increases in response to dehydration and that the RNAi lines may be more drought tolerant than wild type, suggests a possible role for BOLT in drought stress response that leads to early flowering. The MAPK cascade discussed in Chapter 2 is well studied with respect to defense, however it has also been reported that drought can activate MKK1 (Matsuoka et al. 2002; Gao et al. 2008; Kong et al. 2012; Pitzschke et al. 2009). These results, taken together with my Chapter 2 results showing BOLT expression responds to dehydration stress (Fig. 2.3), suggest a possible dual role for BOLT in inhibiting drought stress response, possibly prolonging the life of the plant, and inducing flowering under stressed conditions, thereby increasing the likelihood of a next generation.

BOLT expression may also affect flowering time in response to biotic stresses suggesting a possible role in defense. In Chapter 2 I showed that BOLT expression is regulated in response to flg22, SA and JA, and wounding (Fig 2.3). SA and JA, long
identified as biotic defense-associated hormones, have also been shown to be important regulators of flowering time. These hormones are an important link between defense response and flowering time (Zhai et al. 2015; Van Wees & De Swart 2000; Korves & Bergelson 2003; Lyons et al. 2015; Xue et al. 2013; Martínez et al. 2004). I hypothesized in Chapter 2 that BOLT operates downstream from the MEKK1→MKK1/MKK2→MPK4 pathway (Fig. 2.4). MKK1, MKK2, MPK4, and its substrate MKS1 have all been shown to negatively regulate both SA- and JA-mediated defense (Qiu et al. 2008; Brodersen et al. 2006). The mpk4 mutant accumulates SA and results in constitutive defense gene expression (Qiu et al. 2008).

The four SA-related defense genes discussed in Chapter 2 as possibly regulating BOLT, PAD4, SIZ1, SID2, and NPR1, all have been shown to have roles in flowering time.(LI et al. 2012; G.-F. Wang et al. 2011; J. B. Jin & Hasegawa 2008) NPR1 has been shown to negatively regulate flowering time and an npr1 mutant displays an early flowering phenotype, and like the early flowering phenotype in the BOLT overexpressors, it is independent of the photoperiod (Fig 3.3) (G.-F. Wang et al. 2011). There is evidence that these four genes are also regulated by the same MAPK cascade that affects BOLT’s expression (Qiu et al. 2008; Brodersen et al. 2006; Kapushesky et al. 2011; Petryszak et al. 2013; Petryszak et al. 2016).

Considering that BOLT responds to biotic stress-related treatments, is regulated by genes in several pathways that all have roles in both defense and flowering time, and is potentially an inhibitor of stress response, it seems likely that BOLT has a role in balancing stress response with flowering time.
To further our understanding of BOLT in such a role, we could subject the *BOLT* transgenic lines to biotic stresses such as bacterial and fungal infections and analyze the resulting phenotype. Also, analyzing *BOLT* expression in mutants of *PAD4, SIZ1, SID2*, and *NPR1* as I did in Chapter 2 with the with *mkk1, mkk2*, and *mkk1/2* mutants, could suggest whether BOLT is regulated by one or more of these genes.

**Possible role for BOLT in photosynthesis**

Most of the differences observed between the overexpressing lines and the wild type are apparent in all photoperiods and both light intensities, however, the plants grown in low light are very small and spindly compared to those grown in normal light. The rosettes fall over even before they bolt and the stems are thin and rangy. Since early flowering and other differences occur under all photoperiods and light intensities, and this small/weak phenotype occurs only in low light, it appears the two phenotypes could be the result of different genetic responses.

BOLT must therefore have a role in a pathway that is not required in sufficient light. If this is the case, under normal light there may be no phenotype, but under low light, in which the function is required, the deficiency would be apparent. I discuss one possibility of such a role for BOLT in Chapter 4, where microarray results show that an increase in *BOLT* expression downregulate a significant number of genes involved in cyclic electron flow which has a role in photosynthetic performance (Munekage et al. 2004).

Although it seems probable that the small/weak phenotype is caused by the inhibition of photosynthesis, to determine if it could be a more general stress response,
overexpression lines could be grown in normal light but subjected to other varieties of stress such as cold, dehydration, or pathogen challenge, to see if the small-plant phenotype could be reproduced with different stresses. This could show whether the response is to low light in particular or a response to various stresses.

**Leaf morphology and shade avoidance**

Regulation of leaf flatness was seen in plants overexpressing *BOLT*. Since it was seen under all the light conditions tested we can see that it is not related only to photoperiod or to the light intensity. Flattening of leaves is a well-documented shade avoidance mechanism (Casal 2012; Ciolfi et al. 2013; Nozue et al. 2015). One of the key regulators of shade avoidance is the red/far red photoreceptor PHYB which promotes curled leaves (Kozuka et al. 2013). PHYB has been shown to accumulate in nuclear photobodies in association with certain transcription factors in response to red light (M. Chen 2008; Van Buskirk et al. 2012; Geilen & Böhmer 2015). In Chapter 2 I showed that BOLT::YFP accumulates in some type of nuclear body and referred to publicly available expression data that indicate expression is regulated in a phyb mutant. These data, taken together could suggest that BOLT has a role in the leaf morphology feature of shade avoidance through an association with PHYB. It would be interesting to investigate whether BOLT associates with PHYB in photobodies by co-expressing the tagged genes in onion epidermis to see if the signals overlap. If they did, further testing for physical interaction could be done using BiFC or Co-IP.
Seed size suggests BOLT may affect embryogenesis

One case in which the overexpressing and RNAi lines show an opposite phenotype is in seed size. The overexpressing lines have slightly smaller seeds than wild type and the RNAi lines seed are slightly larger. The seed-size phenotype in the overexpressors could be the result of the reproductive schedule being accelerated, causing not only early flowering, but more rapid seed development. This could mean not enough time for the seed to develop properly. The seeds used in this experiment were T3 and T4 generation, however the measurement was done without regard to the light conditions in which the parent plants were grown. Since this could have an effect on seed size, it would be interesting to grow plants for several generations in consistent conditions to further analyze the phenotype. The GUS staining results showed a dark stained embryo (Fig. 2.1). In addition, as discussed in Chapter 2, BOLT expression is regulated in some genome-wide expression experiments by four key seed-development transcription factors, *LEC1, LEC2, FUS3*, and *ABI3* (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011). This suggests that rather than a general result of accelerated reproduction, BOLT may have a role in seed development in response to stress. I was interested to know if there is evidence in the literature to suggest a connection between BOLT and these genes. The analysis of the *BOLT* promoter sequence discussed in Chapter 2 identified an RY-repeater motif 950 base pairs upstream from the ATG (Yilmaz et al. 2010; Davuluri et al. 2003). *FUS3, LEC2*, and *ABI3* have been shown to bind this sequence (Reidt et al. 2000; Kroj et al. 2003; Mönke et al. 2004). This could either suggest an additional role for BOLT in embryogenesis or for *FUS3, LEC1, LEC2,*
and ABI3 in stress response. A first step to determine if BOLT is in a pathway with these genes would be to obtain the mutants and assess BOLT’s expression in them.

**Methods**

**Plant growth**

Seeds were planted in soil in 3” pots, stratified at 4°C in the dark then transferred to growth chambers. Eight plants of each line were grown under each condition. Plants were grown at 22°C under the following light conditions: Continuous 115µM/m²/sec light, 16 hour 115µM/m²/sec light, 12 hour 115µM/m²/sec light, or continuous 50µM/m²/sec light. A plant was considered to have bolted once the stem measured 1mm.

**Generation of transgenic plants**

Generation of overexpressing lines is described in the Chapter 2 methods section.

To create the RNAi lines, I used the design tool and protocol described at http://wmd2.weigelworld.org (Ossowski et al. 2008; Schwab et al. 2006).

The following primers were used to construct the artificial microRNA:

I 5’-gaTTAACGATACAAACGTACGCGTtctctttgtattcc-3’

II 5’-gaACGCGTACGTTGTATCGTTAAtcaaagagaatcaatga-3’

III 5’-gaACACGTACGTTGTTTCGTTATtcacaggtcgtgatatg-3’

IV 5’- gaATAACGAAACAAACGTACGTGttctacatatatattcct-3’

A-attB 5’-

ggggACAAAGTTTTGTACAAAAAAGCAGGCTTCTgcaaggggattaagtggttaac-3’
B-attB 5’- 
ggggACCACTTTGTACAAGAAGCTGGGTTgcggataacaatttcacacaggaaacag-3’

Gateway cloning was used to combine the resulting plasmid with pDONR-Zeo (Thermo Fisher) to generate the entry clone which was combined with pMDC32 (Curtis & Grossniklaus 2003) resulting in the expression vector 2X35S::amiRNA.

Plant transformation

Plants were transformed using the Clough and Bent method (Clough & Bent 1998). The resulting seeds were surface-sterilized in 25% bleach with 0.01% Triton-X, a surfactant, for 10 minutes then washed six times in sterile, nano-pure water. The sterilized seeds (T1) were plated on 1/2MS medium (pH 5.8) solidified with 0.8% phytoagar supplemented with 30 µg/ml Hygromycin B (hygro plates). The plates were cold-treated at 4°C in the dark for four days then transferred to a long-day growth chamber (115µM/m²s light and 22°C temperature). Tall, green transformed plants (T1, all heterozygous) were selected over stunted pale ones. Identification of homozygous plants was made by plating T2 seeds on hygro plates, collecting the 75% non-wild-type plants, then plating seeds from those plants (T3) on hygromycin plates and choosing the lines from plates in which all plants were tall and dark green (about 1/3 of the plates). T3 homozygous lines were used in experiments.

The following PCR primers were used to confirm the amiRNA in the homozygous lines:

A 5’ – ACAAGTTTGTACAAAAAAGCAGGCTTC – 3’
**qPCR testing of expression levels of RNAi and overexpressing lines.**

Materials, methods, and primers used in the experiments are described in the Chapter 2 Methods section.

**Stress treatments of RNAi plants**

1.5 week-old seedlings were used in treatments which continued for four weeks.

- **cold treatment** – 1.5 week-old seedlings were placed at 10°C and 115μM/m²/sec continuous light.

- **wounding treatment** – Starting with 1.5 week-old seedlings, each leaf was pierced with an 18-guage needle every other day.

- **dehydration treatment** – water was withheld from two-week-old plants for 22 days, after which the plants were watered.

**Seed measurement**

Seeds were fixed to slides (50 per line) using Telesis silicone adhesive and imaged with a light microscope fitted with a Zeiss camera. Seed area was measured using ImageJ. Experiment was repeated twice.
CHAPTER 4: Genome-wide transcription analysis of BOLT

Introduction

In Chapter 2 I investigated BOLT expression in response to different biotic and abiotic stresses and found that BOLT transcript levels increase in response to cold, drought, ABA, wounding, and the flg22 peptide, and decreases in response to SA (Fig 2.3). I also found evidence that BOLT expression is downregulated in the mkk1 mutant and strongly downregulated in the mkk1/mkk2 double mutant (Fig. 2.5). MKK1 and MKK2 are part of a MAP kinase pathway that has been shown to be involved in biotic and abiotic stress signaling both through, and independent of, ROS (Miura & Tada 2014). Therefore, I hypothesized that BOLT is regulated by this MAP kinase pathway and plays a role in response to both biotic and abiotic stress (Fig 2.4).

In Chapter 3 I presented evidence that transgenic lines overexpressing BOLT flower early in comparison to wild-type plants and have distinctly flatter leaves (Figs. 3.3 and 3.7). The RNAi lines flowered somewhat earlier than wild type, but later than the plants overexpressing BOLT (Fig. 3.3). In low light the overexpressing plants are much smaller and spindlier than the wild type, whereas in 12-hour days under normal light, the overexpressing plants are larger than wild type (Fig. 3.4). In low light the RNAi plants were larger than wild type, but in normal light there was no size difference (Fig. 3.4). These results suggest definite effects of BOLT overexpression, affecting both flowering time and growth.

To further investigate the hypothesis that BOLT is in the MEKK1→MKK1/2→MPK4 pathway, I next wanted to know what genes function
immediately downstream of *BOLT* and are potentially regulated by *BOLT*. Genome-wide transcriptional analysis provides a wealth of information on how transcript abundances changes under different conditions or between different genotypes. Thus we decided on a microarray experiment to identify genes regulated by *BOLT*.

We then considered what would be the best comparison to make that would give us the most meaningful information. We hypothesize that *BOLT* is involved in stress responses, however since it responds to many stresses and so do many other genes, doing the experiment with treated vs untreated samples was only briefly regarded. To use treated samples would require data filtering and analysis that would lessen the value of the results and would provide information regarding only one condition. We decided to look at transgenic lines.

The RNAi lines display no obvious phenotype under usual growing conditions, suggesting there may be functional redundancy with one or more genes causing the effects of the knock-down to be masked. It could also be the case that *BOLT* is not essential under normal growing conditions, so using these lines under usual conditions may not provide worthwhile results. An alternative approach was to use an overexpression line (J. Z. Zhang 2003). I had made two dozen transgenic lines that constitutively overexpress *BOLT*, however since they constantly express *BOLT*, using any of those lines would not show genes that had only recently been affected by *BOLT*. In addition, results attributable to the inhibition or enhancement of other transcription factors could be possible as a consequence of *BOLT*’s depletion of available of co-factors.
To avoid the problems associated with using a constitutive overexpressor, I used a transgenic line, created by the TRANSPLANTA consortium (Coego et al. 2014), purchased from the Nottingham Arabidopsis Stock Centre (NASC), that overexpresses BOLT using a β-estradiol-inducible promoter. We then considered whether to use the transgenic line exclusively and compare treated plants to untreated plants, or to compare wild-type to the transgenic line, both treated with the inducer. We chose the second option because there is evidence that β-estradiol affects the expression of some genes (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011). So, I treated wild-type and transgenic plants with the inducer and measured expression over the course of 24 hours using qPCR. BOLT’s expression increased 25-fold in that time (Fig 4.1).

In addition to being able to conditionally over express BOLT, my previous results showing that BOLT-YFP is detectable only in the nucleus of the plant, and showing tissue specificity that agreed with the GUS staining pattern, indicates that BOLT may
Figure 4.1  BOLT expression in wild type and inducible lines

Ten-day-old plate-grown seedlings were treated with 75µM β-estradiol to induce overexpression. RT-qPCR was used to measure BOLT expression 4, 8, and 24 hours after treatment. β-actin and UBI10 were used as reference genes. Data represents three biological replicates and error bars show standard error of the mean.
not be mislocalized in overexpressing plants, decreasing the possibility of off-target
interactions. Using an inducible promoter had two additional advantages. Firstly, BOLT
expression in the inducible line was about 25-fold after 24 hours. This is comparable to
the increase I saw in plants under stress treatments. In the constitutively overexpressing
lines, BOLT expression was approximately 100- to 700-fold higher than wild type
depending on the line, which I thought might lead to less meaningful results caused by
artificially high expression levels. Secondly the ability to induce expression at a
particular time allowed me to focus on the initial changes in gene expression that are
close to a direct result of an increase in BOLT expression, highlighting those genes that
are close to BOLT in the pathway.

Results

Experimental design

In designing the experiment, our aim was to identify genes that BOLT
immediately affects. To determine what time points to sample we considered the results
of two studies, one of which used CHiP-seq followed by RNA-seq to identify ethylene
binding and the related subsequent transcription of downstream genes (Chang et al.
2013). The authors found waves of transcription beginning four hours after binding and
continuing until the end of the experiment, at 24 hours. In the second study, the authors
developed and tested the XVE chimeric transcription activator used in the
TRANSPLANTA lines, showing the increase in transcription of a transgene (GFP) to be
linear between six and 24 hours after induction with some induction as early as one hour
(Zuo et al. 2000). Based on these results, I used samples collected at 0, 8, 12, and 24 hours after induction.

**BOLT expression in inducible line**

To test the expression of the chemically-inducible line, transgenic seeds were grown, together with wild type, on plates under continuous 115µM/m²s at 22°C for ten days. Treatment with inducer was by spraying, with 75µM β–estradiol. *BOLT* expression increased approximately 25-fold over 24 hours in the inducible line, but not the wild type (Fig. 4.1). For the microarray samples, the same growing conditions and treatments were applied. Samples were collected before treatment (0 hr time point), and 8, 12, and 24 hours after treatment, total RNA isolated, and DNase treatment applied as described in Chapter 2. The samples were tested for integrity on a BioRad Experion (Fig. 4.2).

**Microarray data analysis**

Oaklabs, Hennigsdorf, Germany performed the microarray experiment using an Agilent platform and provided us the raw data. Our collaborators at Penn State, Dr. Réka Albert and Dr. Jorge G.T. Zañudo normalized the data using quantile normalization and performed a one-way ANOVA for each gene in each sample type. Using the Storey method, their statistical analysis generated sets of 208 (q<.01) and 1142 (q<.05) genes differentially expressed over the time course and between the wild-type and the induced transgenic line (Storey et al. 2005; Storey et al. 2015). A q-value is the false discovery rate (FDR) adjusted p-value. A notable result was that majority of the differentially expressed genes (62% of the 208 and 61% of the 1142) were downregulated over the
time course and significantly more were downregulated between 12 and 24 hours (84% and 69% respectively) suggesting that BOLT may be a negative regulator of transcription.

Initially I wanted an overall picture of what genes were differentially expressed between the wild type and the induced line to see if the data as a whole suggested a role for BOLT so the first thing I did with the results was to compare the genes differentially expressed per the microarray with the *Arabidopsis* genome as a whole. To do this, I used TAIR Gene Ontology Annotations, which provides functional annotations for sets of genes, to compare the GO terms assigned to the 208 differentially expressed genes (q<.01) in the microarray to the GO terms assigned to the genes in the entire *Arabidopsis* genome (Berardini et al. 2004).

Next I wanted to know if the data contained evidence that any of the genes differentially expressed in the microarray were also regulated by genes in the three groups of potential upstream genes identified in Chapter 2. In that chapter I considered experimental and publically available evidence that MPK4, M KK1 or M KK2, the four embryogenesis genes LEC1, LEC2, FUS3, or ABI3, or the three SA-related genes PAD4, SID2, and NPR1 all regulate or potentially regulate BOLT (Fig. 2.5) (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011).

Then, I wanted to know the identities of genes differentially expressed in the microarray that are related to each other by molecular function, biological process, or cellular component. I did this using the Database for Annotation, Visualization and Integrated Discovery (DAVID), which can be used to identify enriched biological themes and to cluster functionally-related gene groups, I analyzed the gene ontology (GO) terms
in the 1142 differentially expressed genes (q<.05) to identify enriched clusters that may suggest a role for BOLT (D. W. Huang, Sherman & Lempicki 2009a; D. W. Huang, Sherman & Lempicki 2009b)

**BOLT is a transcriptional repressor that regulates chloroplast- and stress-related genes**

Using the Gene Ontology Functional Categorization tool at the TAIR website I compared the GO terms classifications of the 208 differentially expressed genes (q<.01) to the background set of all the *Arabidopsis thaliana* genes (Fig. 4.3). The smaller gene set was used because of the limitations of the TAIR website. The largest category of terms for the microarray set of genes was “response to stress or stimulus”. Thirty-three percent of the differentially expressed genes had terms in this category compared to 24% of genes in the *Arabidopsis* genome. We had expected to see genes related to stress be differentially expressed in our results. The largest differences between the differentially expressed genes and the genome as a whole were in Cellular Compartment ontology terms. We were surprised to see that 30% of the differentially expressed genes had a “chloroplast” ontology term whereas in the total genome, that proportion is only 14%. “Mitochondria” terms are also overrepresented, and “plastid” terms are three times higher in the differentially expressed gene set (Fig. 4.3). Genes with the term “plasma membrane” are more than twice as prevalent in our microarray results as in the genome. This is an overall analysis that gives us an idea of what types of genes BOLT regulates. It indicates regulation preferentially targeted to organelles and the plasma membrane.
Although surprising at first, upon consideration, these are areas of the cell which are active in defense and abiotic stress thus the results are reasonable.

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Figure 4.2 Quality assessment data for RNA microarray samples

Genes upstream from BOLT are known to regulate genes differentially expressed in the microarray

Our hypothesis is that BOLT responds to stress and is downstream of the MEKK1 → MKK1/MKK2 → MPK4 cascade so I next wanted to know if there were genes differentially expressed in the microarray that are also regulated by genes in the MAPK cascade. I used European Bioinformatic Institute’s (EBI) Expression Atlas (Petryszak et al. 2016; Kapushesky et al. 2011) to query how many of the 208 differentially expressed genes (q < .01) were shown in its database to have altered expression (>2-fold and p > .05) in the mkk1, mkk2, or mkk1/2 mutants. Sixty-six genes of the 208 were also regulated in mkk1 or in the double mutant. Fifty-seven were downregulated and 9 were upregulated. To get an idea if this was significant, I performed the same query with four different random samplings of 208 Arabidopsis gene IDs obtained using atgenie.org/random-list (Sundell et al. 2015). The number of random genes regulated in the mkk1, mkk2, or mkk1/2 double mutant ranged from 29 to 34 with an average of 31 suggesting 66 genes is significant. This further supports that BOLT may operate downstream of the MEKK1 → MKK1/MKK2 → MPK4 pathway.

In Chapter 2 I noted that BOLT is negatively regulated in the lec1, fus3, lec2, and abi4 mutants (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011)(genevestigator.com). These genes are described as master regulators of embryo development, and control the biosynthesis and accumulation of seed storage proteins (Harada 2001; Kroj et al. 2003). Of the 208 (q < .01) genes differentially expressed in our microarray, 60 (35 downregulated, 25 upregulated), 53 (24 downregulated, 29 upregulated), 51 (17 downregulated, 34 upregulated), and 21 (3 downregulated and 18
upregulated) are regulated in the *fus3*, *lec1*, *abi3*, or *lec2* mutants respectively. This suggests a genetic connection between BOLT and these genes, but the nature of the relationship is not clear.
Figure 4.3  GO categories of differentially expressed genes compared to genome as a whole. Gene Ontology categories in genes differentially expressed in microarray (q<.01) and the Arabidopsis genome using Gene Ontology at TAIR (Arabidopsis.org).
In light of the interesting results showing that a significant number of genes differentially expressed in the microarray are apparently regulated by M KK1/M KK2 or the embryogenesis genes, I was surprised to find that is not the case for the SA-related genes, \textit{PAD4}, \textit{SID2}, and \textit{NPR1}. Only \textit{SID2}, important in SA accumulation, regulated any of the differentially expressed genes and only 20 of them (Dewdney et al. 2000; Nawrath & Métraux 1999). This suggests that these genes act on \textit{BOLT} outside of the MAPK pathway.

**GO terms analysis of differentially expressed genes using DAVID**

To assess whether the genes differentially expressed between wild type and the inducible line may be related functionally and to highlight probable biological themes represented in the data I used the functional annotation tool of Database for Annotation, Visualization and Integrated Discovery (DAVID) to cluster the 1142 differentially expressed genes (q<.05). The DAVID software clusters genes based on the degree of similarity between their respective sets of annotation terms (D. W. Huang, Sherman & Lempicki 2009a; D. W. Huang, Sherman & Lempicki 2009b). The clusters are ranked using a calculation based on the p-values of each term’s inclusion in the cluster. Table 2 shows the eight clusters of genes differentially expressed in the microarray that have the highest enrichment scores. DAVID does not create any of the annotation content, its sources are primarily NCBI Entrez Gene (www.ncbi.nlm.nih.gov/Entrez/) and UniProt (www.pir.uniprot.org/).

The cluster of terms with the highest enrichment score also represented the largest number of genes; 222 of the 1142 genes (19%) had “chloroplast” as a GO term. Of these
182 or 82% were downregulated. Chloroplast genes are overrepresented 1.5- to 2-fold in the differentially expressed gene set. The next three clusters were also related to chloroplasts. Twenty-four genes respond to light, 21 are involved in starch biosynthesis and maltose metabolism, and 21 genes have the term “photosystem II assembly”. These genes are involved in primary and secondary metabolic pathways as well as in photosynthesis and carbon metabolism and are overrepresented 2.4 to 4-fold in the set of differentially expressed genes.

The next two clusters contain the most statistically overrepresented groups of genes compared to the genome as a whole, with 4 to 23-fold enrichment over a random sample of the same number of genes. These two clusters are particularly interesting in that each is comprised of genes only from a specific complex, the glycine decarboxylase complex (GDC), or the NAD(P)H dehydrogenase complex (NDH). DAVID identified four GDC genes and eight NDH genes. Further review of the differentially expressed genes revealed that BOLT regulated three more genes encoding GCD proteins. This total of six genes encodes all of the proteins of the GCD. Six additional genes encoding subunits and ancillary proteins of the NDH were also differentially expressed bringing the total to fourteen differentially expressed NDH complex genes.

The GDC system, comprises four proteins, which together, are the mitochondrial component of the photorespiratory system, and function in the glycine to serine interconversion reactions in that system (Fig 4.4) (Timm et al. 2012). In Arabidopsis, the four proteins are encoded by eight genes, two genes each for P-protein (At4g33010 and At2g26080) and L-protein (At3g17240 and At1g48030), three genes for H-protein
(At2g35370, At2g35120 and At1g32470) and one gene for T-protein (At1g11860) (Bauwe 2003; Hasse et al. 2013; Buchanan et al. 2015). Six of these eight genes,
Table 2  GO terms clusters of differentially expressed microarray genes with highest enrichment scores (q<.05) using DAVID (david.ncifcrf.gov).

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representing all of the proteins, are downregulated in the microarray data suggesting BOLT inhibits the activity of the GDC system (Fig. 4.5). The strongest regulation occurring between 12 and 24 hours after BOLT induction.

The NDH complex is located in the stroma-exposed thylakoid membrane and functions in one of the two cyclic electron flow (CEF) systems, which route electrons around photosystem I (PSI) resulting in reduction of the plastoquinone and acidification of the lumen (Fig. 4.6) (Suorsa, 2015). PGR5, a key component of the second CEF system, the PGRL1-PGR5 complex, is also downregulated in the microarray results, as is ferredoxin (FD1), which accepts electrons from PS1 and routes them into CEF. CEF is much less well understood than the linear electron flow of photosynthesis, however evidence of roles in response to environmental changes and in development has recently been demonstrated (Suorsa 2015).

There are twelve genes encoding NDH complex subunits that are differentially expressed in the microarray results: NDHA, NDHF, NDHM, NDHN, NDHO, NDHT, PNSL1, PNSL2, PNSL3, PNSL4, PNSB1, PNSB2, as well as several genes, including CRR3 and CRR7 shown to be involved in the assembly of the complex (Ifuku et al. 2011) (Fig. 4.7). These genes were all downregulated in the microarray experiment and again most of the difference is between 12 and 24 hours after the induction of BOLT (Fig. 4.8).
Figure 4.4 Models of the Glycine Cleavage and Photorespiration systems

Figure 4.5  GDC genes are downregulated in induced line

Comparison of GDC genes expression in microarray indicating downregulation over the time course, particularly at 24 hours. Vertical axis is normalized expression and horizontal axis is hours after treatment with 75μM β-estradiol inducer. A, wild type samples. B, induced samples.
The last significant cluster we considered contains 66 (p<.05 for inclusion in the cluster) genes with “photosynthesis”, “response to cold”, “MAPK cascade”, JA and SA signaling or biosynthesis terms, and a number of pathogen and defense response terms (Table 2). This cluster adds support to the hypothesis that BOLT has a role in abiotic and biotic stress responses and reinforces the qPCR data showing BOLT responds strongly to cold temperature (Fig. 2.3).

The results indicating that BOLT may regulate genes in the chloroplast and other plastids in its role in biotic and abiotic stress signaling may have been the most valuable information to come out of this project. Before doing the microarray experiment, we had evidence that BOLT has a role in both biotic and abiotic stress response as do other AP2/ERF transcription factors, and we could see a phenotype in the overexpressing plants, however, I think no other experiment we could have done would have demonstrated so clearly that we need to look at the chloroplast and other plastids to elucidate BOLT’s function.
Figure 4.6 Model of the Cyclic Electron Flow system

Based on representation by Yamamoto et al (Hiroshi et al. 2016). Cyclic Electron Flow (CEF) and Linear Electron Flow (LEF) are indicated by red and blue arrows respectively. CEF systems, NDH and PGR5/PGRL1, are designated by a red border.
Figure 4.7 Model of NDH subcomplex composition

(Ifuku et al. 2011). Plastid-encoded subunits are in red and nuclear-encoded subunits are in black. Genes differentially regulated in the microarray experiment are outlined.
Figure 4.8  CEF genes are downregulate in induced line in microarray

Comparison of CEF genes expression in microarray indicating downregulation over the time course, particularly at 24 hours. Vertical axis is normalized expression and horizontal axis is hours after treatment with 75µM β-estradiol inducer. A, wild type samples. B, induced samples.
**Genes co-expressed with BOLT are regulated by the same MAPK cascade**

Using the 208 genes that are differentially regulated (q<.01) as nodes, our collaborators generated a co-expression network to understand and visualize the connections between and among *BOLT* and those genes. They connected each pair of genes with an edge if the average time course has a Pearson correlation coefficient greater than 0.95 (positive regulation) or lower than -0.95 (negative regulation) (Fig. 4.9). The co-expression network, being a correlation network, does not have an inherent directionality, however in this network, we see that most of the genes that are differentially expressed only at the 24hr time point are more than three edges away from *BOLT*, and 90% of the genes that are three or fewer edges from *BOLT* are differentially expressed at the 8hr time point. In addition, all but two of the genes that are differentially expressed at all time points are three or fewer edges away from *BOLT*. All of this suggests that the network is consistent with a directionality emanating from *BOLT* and the nodes that are one, two, or three edges away in the co-expression network are likely directly, or close to directly, downstream from *BOLT*.

Nine genes are one edge away from *BOLT*, seven of which are downregulated. Three of these genes are also regulated in the mkk1/2 double mutant (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011) (genevestigator.com).
Figure 4.9  Co-expression diagram including 208 differentially expressed genes

A red edge indicates negative regulation. A gray edge indicates positive regulation. The genes are colored according to the time point at which they are differentially expressed: green, 8hr; yellow, 12hr; dark blue, 24hr; aqua, all time points. Genes that are within three edges from BOLT have a thick black outline.
Four have abiotic or biotic stress GO terms, and all but one is regulated at 8 hours with three regulated over the entire 24 hours. The nine genes have, for the most part, not been studied at all. BBX32 was shown to antagonize HY5, and is proposed to function downstream of photoreceptors modulating light responses (Holtan et al. 2011). A number of the genes have defense-related GO terms assigned computationally. The most common being “response to fungus”, “metal ion binding”, “response to oxidative stress”, and “transcription regulation”. Two edges away from BOLT are 34 genes, 16 of which are regulated in the mkk1/2 double mutant, and three edges away are 115 genes, 47 of which are regulated in the mkk1/2 double mutant (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011) (genevestigator).

Taken together these microarray results suggest that BOLT regulates genes that have roles in defense, light, and cold temperature responses. Many of the genes BOLT regulates are in the chloroplast and other plastids. Two complexes in particular are downregulated by BOLT, the GDC and the NDH. This is particularly exciting as the work to date that has been done on these systems is limited and these results could help to move that part of the field forward.

**Discussion**

The results of our microarray experiment have given us much valuable information about the transcription regulation role BOLT has in *Arabidopsis*. I was able to tie differentially expressed genes to the upstream MAPK cascade as well as to two other groups of genes, that potentially regulate BOLT, LEC1, LEC2, EBI3, and FUS3 (embryogenesis genes), and PAD4, SID2, and NPR1 (SA-related genes). This expands
our hypothesis significantly to include these genes acting through \textit{BOLT} to regulate development, as well as cold and defense responses.

Analysis of GO terms of the differentially expressed genes highlights BOLT’s role in the GDC, and possibly photorespiration as a whole, cyclic electron flow, photosynthesis, and defense, and suggests that many of the genes BOLT regulates are in the chloroplast and other plastids. In addition, the downregulation of CEF by the plants overexpressing BOLT supports the phenotypes we see in the overexpressing plants and is the opposite of that in the RNAi lines, which are slightly larger than wt, suggesting a dual role in low light and stress response (Fig. 3.5).

This discussion will mirror the results section. First I will discuss the three groups of genes, MAPK, embryogenesis, and SA-related that regulate BOLT (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011). The results of these analyses expand and support the hypothesized pathway. Next I will talk about the analysis of the microarray results as a whole using GO terms analysis. The resources at TAIR provide an interesting overall comparison between my data and the \textit{Arabidopsis} genome, and the tools associated with the DAVID database highlight Biological Processes and Cellular Components in which the differentially expressed genes are enriched. Prominent categories in these analyses are discussed in detail.

\textbf{Differentially expressed genes also regulated by MAPK, SA-related, and embryogenesis genes}

The microarray results show that 66 of the 208 differentially expressed genes (p<.01)are also regulated in one or both of the mkk mutants, more than double the
number that would be expected based on a test using four random sample gene sets (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011). This evidence supports my hypothesis that BOLT acts downstream of the MEKK1 → MKK1/MKK2 → MPK4 cascade. The MEKK1 → MKK1/MKK2 → MPK4 pathway has been shown to negatively regulate defense responses by repressing cell death and immune responses, however the mechanism is not clear (Kong et al. 2012). Mutants of the pathway accumulate high levels of SA, and exhibit enhanced pathogen resistance (Petersen et al. 2000; Ichimura et al. 2006; Nakagami et al. 200;) Suarez-Rodriguez et al. 2007). It is known that MPK4 downregulates SA accumulation and represses immune function and the microarray array results show that BOLT may also have a role in downregulating defense (Petersen et al. 2000)(Table 2). The MEKK1 → MKK1/MKK2 → MPK4 cascade is downstream from PAMP receptors, and MKK1 is required for full activation of MPK4 in response to flg22 treatment which also upregulates BOLT expression (Fig 2.3) (Droillard et al. 2004). Taken together this suggests that BOLT has a role in defense and immunity through the MAPK pathway. To further understand its function, we would want to measure SA levels in the overexpressing and RNAi plants as well as study BOLT’s responses to a wider variety of pathogens and/or elicitors in wild-type and the transgenic lines especially, because of the SA accumulation in the MAPK mutants, in the BOLT-RNAi lines.

The MAPK cascade also has a role in cold, drought, and salinity responses, particularly through MKK2 (Furuya et al. 2014; Teige et al. 2004; Ichimura et al. 2000). Although MKK1 and MKK2 appear to act redundantly, generally MKK1 is activated by biotic stress while MKK2 has been shown to respond to abiotic stress, particularly cold
temperatures (Furuya et al. 2014; Qiu et al. 2008). BOLT responds to both biotic and abiotic stresses and is downregulated in both the mkk1 and mkk2 mutants, but more so in mkk1 (Figs. 2.3 and 2.5). Our microarray analysis indicates that thirty-five differentially expressed genes have a “cold” GO term, this further suggests that BOLT could act through both MKK1 and MKK2 (Table 2).

The MEKK1→MKK1/MKK2→MPK4 cascade also plays an essential role in ROS metabolism (Nakagami et al. 2006; Pitzschke et al. 2009). H$_2$O$_2$ accumulates in mekk1 and mpk4 mutants and it also activates MEKK1 in protoplasts (Nakagami et al. 2006). Because the MEKK1 protein level is also increased by H$_2$O$_2$, the MEKK1 cascade may be part of a feedback loop that regulates and responds to ROS levels. ROS scavenging mechanisms are disrupted in mekk1, mkk1/2, and mpk4 mutants (Pitzschke et al. 2009). These MAPK cascades are controlled by both SA and ROS (Pitzschke et al. 2009). Because oxidative stress is a common response to biotic and abiotic stresses, ROS homeostasis is a convergence point at which to evaluate the plant stress status (Miura & Tada 2014). It has been shown that this MAPK pathway regulates ROS homeostasis (Pitzschke et al. 2009). Because BOLT is regulated by the MAPK cascade and the cascade is activated by ROS, it would be interesting to measure ROS, especially H$_2$O$_2$, levels in the BOLT transgenic plants as well as to measure BOLT’s response to exogenously applied ROS.

More surprising and less clear than BOLT regulating defense-related genes is that BOLT regulates genes that are also regulated by embryogenesis-related transcription regulators. Embryos stained very dark in the GUS experiments indicating high expression in that tissue (Fig. 2.1). Four well-studied genes $FUS3$, $LEC1$, $LEC2$, and $ABI3$ are
central transcription regulators of embryo development, where they control the biosynthesis and accumulation of seed storage proteins (Harada 2001; Kroj et al. 2003). As discussed in Chapter 2, BOLT is downregulated in embryos of all four mutants, upregulated in a FUS3 overexpressor, and has an RY motif in its promoter 970 nt upstream from the translation start site (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011; Palaniswamy et al. 2006; Yilmaz et al. 2010; Davuluri et al. 2003). FUS3, LEC2, and ABI3 each has a B3 DNA-binding domain that has been shown to bind the highly conserved RY motif (Reidt et al. 2000; F. Wang & Perry 2013). In addition, the embryogenesis genes and BOLT all respond to ABA (Fig. 2.3).

Most of studies of these four genes have been focused on their roles in embryogenesis, however recently, involvement in post-embryotic development has been demonstrated (Yamamoto et al. 2010; F. Wang & Perry 2013). A 2010 microarray study using a FUS3 overexpressor and fus3 knock-down lines that suggest the gene’s role may be more diverse than its well-researched function in embryogenesis and seed maturation (Yamamoto et al. 2010; Wang & Perry 2013). Yamamoto et al. (2010) found regulation of genes that are expressed in leaves, shoots, and flowers well represented in the set of differentially expressed genes and showed that genes controlled by FUS3 are not confined to embryogenesis, but include genes involved in response to stimuli, including light intensity, temperature, and abiotic stress, as well as phloem loading, photosynthesis and the production of secondary metabolites, particularly hormone biosynthesis, (Yamamoto et al. 2010). In a ChIP-chip experiment to examine what sequence(s) FUS3 binds, transcription factors were significantly overrepresented (Wang & Perry 2013).
A 2008 study compared the fus3-3 mutant used in the above studies as well as many more, to a fus3 T-DNA line, found differences in the phenotypes, and concluded that the fus3-3 mutation induces pleiotropic effects due to a truncated gene product. The conclusion was that FUS3 function is restricted to embryogenesis (Tiedemann et al. 2008). This should be considered in any investigation of BOLT with regard to FUS3, however, the fact that the FUS3 overexpressing line showed opposite gene expression to fus3-3 corroborated the results in the Yamamoto paper (Yamamoto et al. 2010).

LEC1 too, is well known to have roles in embryogenesis and seed maturation, but has very recently also been shown to be a co-activator of PIF4 in transcriptional regulation during postembryonic growth (Huang et al. 2015; Meinke et al. 1994; West et al. 1994; Harada 2001). PIF4 is a phytochrome mediating factor that integrates environmental signals in the coordination of stress vs. growth responses (Koini et al. 2009). Junker et al also suggests a post-embryonic role for LEC1 as a result of ChIP-chip using two-week-old seedlings showing target genes involved in response to hormone stimulus, developmental processes, response to light stimulus, light harvesting, chlorophyll binding, DNA binding, and transcription factor activity, demonstrating that LEC1 is active in post-embryonic plants (Junker et al. 2012).

Since these genes appear to regulate a significant number of genes that are differentially expressed in our microarray, a regulatory pathway from these genes through BOLT to the downstream genes could be a possibility (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011). Considering Yamamoto’s and Huang’s results together with ours, if BOLT is in a pathway with these four embryo/seed-related genes it would
support this latest research into roles for *FUS3* and *LEC1* in biotic and abiotic stress responses (Yamamoto et al. 2010; Huang et al. 2015).

In addition to *MKK1/MKK2* and the embryogenesis genes, *BOLT* is also regulated by a trio of SA-related genes, *PAD4*, important for SA signaling, *SID2*, which has a role in SA accumulation, and *NPR1*, a key regulator of the SA-mediated systemic acquired resistance pathway (Jirage et al. 1999; Wildermuth et al. 2001; Villajuana-Bonequi et al. 2014; Dong 2004; Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011)(genevestigator). It was shown that the MAPK cascade that includes MEKK1, MKK1/2, and MPK4 regulates SA accumulation in which SID2 plays a key role (Pitzschke et al. 2009). Their effect on *BOLT* is opposite to that of the embryogenesis genes (*FUS3, LEC1, LEC*, and *ABI3*), and *MKK1/MKK2*, as *BOLT* expression increases in the *pad4, sid2*, and *npr1* mutants.

Because it was the case that a significant number of the differentially expressed genes were also regulated by the MAPK and embryogenesis genes, I expected to see some regulated by *PAD4, SID2*, and/or *NPR1*. However, this was not the case. Of the 208 differentially expressed genes I found none to be regulated in the *pad4* or *npr1* mutants and only 20 genes regulated in the *sid2* mutant (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011) and genevestigator). The reason for this could be the opposite regulation. *BOLT* is downregulated by these genes so we may not see their effects when it is upregulated as it was in our microarray experiment. This hypothesis could be tested by doing the same microarray experiment as we have done, but including one or more of the RNAi lines. If the above explanation is correct, we might expect to see in the results genes downstream from *PAD4, SID2*, and *NPR1*. 
It is clear that from our results that SA has a role in *BOLT* expression and that BOLT likely has a role in SA biosynthesis, accumulation, and signaling (Fig. 2.3) (Table 2). Of the 208 differentially regulated genes in the microarray 85 are shown to be regulated by exogenous application of SA, in genome-wide transcription experiments (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011). Of those, 27% are upregulated and 73% are downregulated. In addition, in the GO terms analysis using DAVID, 37 genes are identified with a GO term containing “salicylic acid” (Table 2) (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011).

**GO terms analysis strongly suggest light- and defense-related roles for BOLT**

GO terms analysis is an exceedingly useful tool for making sense of large sets of genes. The aim of the Gene Ontology project has been to provide standard language for descriptions of gene products (Ashburner, Ball, Blake, Botstein, Butler, Cherry, Davis, dolinski, et al. 2000; Harris et al. 2004). Ontology terms are assigned based on various information including experimental evidence and computational analysis evidence. Not all genes have been assigned any GO terms, however many genes have multiple terms assigned both manually and electronically. GO term analysis is extremely useful in understanding the functional implications of a large set of genes with large numbers of annotations.

Using the functional annotation tool of the Database for Annotation, Visualization and Integrated Discovery (DAVID) to categorize the differentially expressed microarray genes (p<.05), the four most enriched clusters indicate that a large number of these genes are chloroplast- and photosynthesis-related. The top category is “chloroplast” and
contains 222 genes. The following three categories contain light- and photosynthesis-related terms with 54 unique genes between the three clusters (Table 2). This was initially surprising, however chloroplasts are highly responsive to environmental cues and key in balancing the competing energy requirements of growth and development with those necessary to respond to environmental and defense challenges (Muhlenbock et al. 2008) (Huot et al. 2014) (Kangasjarvi et al. 2012). In Chapters 2 and 3 we have seen that BOLT responds to biotic stress and that overexpression results in a significant light intensity-related phenotype (Figs. 2.3, 3.5, and 3.6). Thus, it should not be a surprise that BOLT would regulate chloroplast and light-related genes.

ROS, hormones and other secondary metabolites are produced in the chloroplasts, and are available for a rapid and immediate response to abiotic and biotic stress as well as for longer-term altered gene expression response (Peterhansel et al. 2010).

For example, functional chloroplasts are required for the hypersensitive response, and P. syringae treatment during the daytime causes a more vigorous defense response than nighttime treatment demonstrating a connection between light and defense (Delprato et al. 2015). The results in Chapter 2 and Chapter 3 indicating BOLT may localize to some type of nuclear body, possibly a photobody, and that the overexpression of BOLT causes a significant low-light phenotype suggest that further investigation into BOLT’s regulation by light may result in a role for BOLT in both light and defense. In addition, investigation of the promoter sequence including 3kb upstream of BOLT shows quite a few light-related DNA-binding sites suggesting BOLT may be regulated by proteins responsive to light (Table 1). An interesting question these results raise is whether there
is an observable chloroplast phenotype or a difference in chlorophyll content between wild type and either the overexpressing or RNAi lines.

**Similar results with another AP2/ERF gene**

I was interested to know if genes regulated by other AP2/ERF transcription factors might have some similar GO annotations as genes regulated per our microarray. To find out, I searched publicly available datasets for genome-wide experiments similar to our microarray that compared gene expression of upregulated AP2/ERF transcription factors to wild-type expression. I found only one, but it is similar to our experiment and the results are very interesting. It is a microarray experiment that included data comparing an ERF104 overexpression line to wt (Table 3). I used DAVID to do a functional analysis of GO terms associated with this data, as I did with our data, and found results that had some striking similarities to the BOLT data, especially in the top three categories (david.ncifcrf.gov). The top cluster is the same as in the BOLT analysis, “chloroplast”, the second is identical to the third cluster in the BOLT analysis and includes “starch biosynthetic process”, “maltose metabolic process”, and “positive regulation of catalytic activity”. The third category is similar to category #8 in the BOLT analysis, including genes related to defense, SA, JA, and MAPK. There are other similar clusters, but there are also divergent ones. Interestingly, the resulting paper describes a stress-related role for ERF104 and demonstrates physical interaction between ERF104 and MPK6 (Timm et al. 2013)
Table 3  GO terms clusters of differentially expressed genes in ERF104

Overexpression compared to wild type (david.ncifcrf.gov).

<table>
<thead>
<tr>
<th>Enrichment Score</th>
<th>Category</th>
<th>Term</th>
<th>Number of genes</th>
<th>p-value</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>57.15</td>
<td>Chloroplast</td>
<td>GO:0009507~chloroplast</td>
<td>699</td>
<td>9.99E-76</td>
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<tr>
<td>35.22</td>
<td>Photosynthesis</td>
<td>GO:0019252~starch biosynthetic process</td>
<td>104</td>
<td>3.13E-43</td>
<td>4.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GO:0000023~maltose metabolic process</td>
<td>88</td>
<td>2.20E-42</td>
<td>4.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GO:0043085~positive regulation of catalytic activity</td>
<td>54</td>
<td>3.13E-22</td>
<td>4.32</td>
</tr>
<tr>
<td>32.86</td>
<td>Defense</td>
<td>GO:0010363~reg. of plant-type hypersensitive resp.</td>
<td>163</td>
<td>5.19E-56</td>
<td>3.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GO:0006612~protein targeting to membrane</td>
<td>163</td>
<td>3.65E-52</td>
<td>3.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GO:0043069~negative reg. of programmed cell death</td>
<td>97</td>
<td>3.50E-46</td>
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<tr>
<td></td>
<td></td>
<td>GO:0031348~negative regulation of defense response</td>
<td>122</td>
<td>5.03E-43</td>
<td>3.85</td>
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<tr>
<td></td>
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<td>GO:0005623~cell</td>
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<td>7.44E-43</td>
<td>2.34</td>
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<tr>
<td></td>
<td></td>
<td>GO:0009697~salicylic acid biosynthetic process</td>
<td>98</td>
<td>6.91E-36</td>
<td>3.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GO:0009862~SAR, SA-mediated signaling pathway</td>
<td>105</td>
<td>3.93E-33</td>
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<tr>
<td></td>
<td></td>
<td>GO:0000165~MAPK cascade</td>
<td>104</td>
<td>2.74E-31</td>
<td>3.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GO:0042742~defense response to bacterium</td>
<td>125</td>
<td>4.51E-30</td>
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<tr>
<td></td>
<td></td>
<td>GO:0009867~JA-mediated signaling pathway</td>
<td>107</td>
<td>9.18E-30</td>
<td>3.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GO:0010310~reg. of hydrogen peroxide met. process</td>
<td>73</td>
<td>1.00E-21</td>
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</tr>
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<td></td>
<td></td>
<td>GO:0009595~detection of biotic stimulus</td>
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<td>7.14E-19</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>GO:0050832~defense response to fungus</td>
<td>140</td>
<td>2.65E-15</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GO:0043900~regulation of multi-organism process</td>
<td>40</td>
<td>7.06E-14</td>
<td>3.73</td>
</tr>
</tbody>
</table>
ERF104 is an AP2/ERF, but it is not in the same sub-family as BOLT. It has one AP2 domain as does BOLT. ERF104 has been shown to increase expression extremely rapidly when light is increased from low to high intensity suggesting a rapid transcription mechanism or sequestered mRNA in an undetectable state (Moore et al. 2014). The interaction mentioned above between ERF104 and MPK6 is disrupted with the application of flg22 peptide (Bethke et al. 2014; Bethke et al. 2009). This is similar to the MPK4-MKS1-WRKY33 association discussed in Chapter 2 suggesting it could be that defense-related transcription factors, including BOLT, are sequestered in the nucleus for swift deployment should a stress cue arrive.

Although this evidence is the result of examining only one other gene, it is very interesting that the most enriched clusters in both microarrays are chloroplast, photosynthesis, and defense-related, and that ERF104 expression is shown to increase rapidly under strong light. These results could suggest a role in chloroplast/defense signaling for AP2/ERF transcription factors through MAPK pathways and can serve as a direction in which to take further research. As genome-wide analyses come down in price, there will be more of this type of data available that can be used to further the field at a lower cost.

**BOLT may have a role in photorespiration**

In our microarray results, the genes that encode all four proteins comprising the GDC of the mitochondrial photorespiratory system are downregulated (Fig 4.5). The GDC is a key component of the photorespiratory system that impacts plant growth and response to pathogens and other environmental conditions. The evidence suggests that
BOLT’s inhibition of the GDC influences stress signaling through redox status, metabolite concentration, and plant growth in low light conditions.

Rubisco can bind either CO$_2$ or oxygen. CO$_2$ binding precedes its entry into the Calvin-Benson Cycle, and O$_2$ proceeds to photorespiration (Fig 4.4). Oxygen is bound approximately one third as often as CO$_2$ under normal growing conditions so photorespiration is an significant set of ongoing reactions in the cell (Timm et al. 2012; Peterhansel et al. 2010). Under light conditions the proteins involved in photorespiratory reactions comprise about half of the proteins in the mitochondria, another indication that photorespiration is a key process (Douce et al. 2001). Recent research has redirected the focus from photorespiration as a wasteful system necessary to accommodate the high concentration of oxygen in the atmosphere, to photorespiration as the major producer of H$_2$O$_2$ and a key system contributing to the production of primary and secondary metabolites, although a decrease in photorespiration under low light and low oxygen environments supports a role in protecting plants against oxidative photodestruction resulting from a combination of high light and high oxygen (Bauwe et al. 2012; Peterhansel et al. 2010; Foyer et al. 2009; Davies, 1980). These processes have a major influence on the cellular redox status and on multiple signaling pathways, particularly those governing hormonal control of growth through photosynthesis, and environmental and defense responses under stress conditions (Foyer et al. 2009; Timm et al. 2012). Because of its participation in multiple pathways, H$_2$O$_2$ is especially suitable for mediating crosstalk between different resistance mechanisms (Neill et al. 2002).

The GDC system represents the major mitochondrial component of the photorespiratory pathway. Very briefly, the GDC converts glycine to serine, CO$_2$ and
NH$_3$(Bauwe 2003; Engel et al. 2007; Palmieri et al. 2010). What is not well understood is how the GDC is regulated within the photorespiratory system and by photosynthesis, or its overall physiological role in the plant (Timm et al. 2012). Both glycine and serine concentrations exert feedback which affects the GDC, photorespiration, and photosynthesis but though these affects are known the mechanisms are not clear (Timm et al. 2012). The CO$_2$ and NH$_3$ produced in the GDC reactions are used in the biosynthesis of secondary metabolites, some of which play key roles in abiotic stress response and defense and Timm et al suggest that serine has a signaling role in photorespiration-related transcription (Sørhagen et al. 2013; Timm et al. 2013). All of the proteins in the GDC are downregulated in our microarray suggesting BOLT affects both photorespiration and photosynthesis (Sørhagen et al. 2013; Palmieri et al. 2010; Wingler et al. 2000; Mouillon et al. 1999; Engel et al. 2007).

GDC is a system of three enzymes (L-protein, T-protein, and P-protein) and one additional protein (H-protein) that shuttles an intermediary between the enzymes(Timm et al. 2012)(Fig. 4.4). Engel et al suggest the GDC is required for survival as a double mutant lacking GDC activity dies at the cotyledon stage, but this is the only study that has shown that (Engel et al. 2007). It is also suggested that GDC activity could be a signal in a regulatory network that adjusts carbon flux through the Calvin-Benson cycle in response to photorespiration, however the jury is still very much out on the physiological roles of the reaction in photorespiration in general and of the GDC complex specifically although it is clear that inhibiting photorespiration reduces photosynthesis and vice versa (Timm et al. 2015 and 2012). This demonstrates one example of coordination between photosynthesis and stress responses. All four of these proteins are
encoded by genes that were downregulated in the microarray results (Fig. 4.5). This suggests that BOLT may have a role in the regulation of photosynthesis through photorespiration.

Although the GDC has not been very well studied, there is some evidence that GDC is involved in responses to abiotic and biotic stress, and light. GDC activity is inhibited by inducers of ROS, such as the bacterial elicitor harpin, wounding, cold temperature, dehydration, and mutant genotypes that result in high ROS levels, mostly conditions in which *BOLT* is upregulated (Fig. 2.3) (G.-T. Huang et al. 2011; Palmieri et al. 2010; Taylor et al. 2002; Hoffmann et al. 2013). In publicly available microarray results the GDC genes are also shown to be regulated by additional stresses including viruses, insect feeding, fungus, phosphate deprivation and low light (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011). All six of the GDC genes downregulated by *BOLT* induced overexpression were also downregulated in the mkk1/mkk2 double mutant and in the mpk4 mutant. (Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011) genevestigator.com). This suggests that we could expand our hypothesized pathway that the MAPK cascade induces *BOLT* expression, to include the subsequent downregulation of GDC genes in response to abiotic and biotic stress.

GDC proteins are also strongly light regulated, and compromising photorespiration impairs photosynthesis (Foyer et al. 2009; Timm et al. 2013; Timm et al. 2012). Rosettes of plants overexpressing *BOLT* are similar to wild type in size in normal light, however in low light they are much smaller. I suggest that the GDC is necessary in low light conditions and the inhibition by BOLT overexpression together with low light reduces photorespiration to such a level that photosynthesis is severely curtailed. It could
be that inhibiting GDC impairs photosynthesis enough to impact the size of the overexpressors under low light. Conversely, the RNAi lines, in which BOLT is knocked down almost completely, grow larger in low light because the GDC is necessary and there is no BOLT to downregulate it. These results suggest that BOLT’s role in downregulating the GDC could be in response to abiotic or abiotic stresses signaled via the MEKK1→MKK1/MKK2→MPK4 pathway and that the low light phenotype suggests that the GDC is not required in normal light but is required in low light.

Another GDC phenotype that corresponds to plants overexpressing BOLT is that plants engineered to bypass much of the photorespiratory system, including the GDC are larger than wild-type plants in short days under normal light intensity (Fig. 4.10) (Kebeish et al. 2007; Peterhansel et al. 2010). This is the same phenotype we see in BOLT overexpressors under similar conditions, suggesting the overexpression of BOLT does in fact downregulate the GDC.

In addition to the six GDC genes in the mitochondria, four photorespiratory genes in the peroxisome are also downregulated in the microarray, GOX1, GOX2, HPR1, and OPR3. The peroxisome is the central \( \text{H}_2\text{O}_2 \) producing organelle of photorespiration and these genes were shown to have roles in defense and immunity through ROS signaling further suggesting a role for BOLT in regulating biotic stress signaling(Sørhagen et al. 2013). To provide further evidence that GDC genes are in a pathway with BOLT and the MAPK genes, a qPCR experiment could be performed using the mkk1/2 double mutant plants, and the BOLT overexpressing and RNAi lines and primers for the GDC genes.
BOLT may have a role in cyclic electron flow

In our microarray results, genes in both cyclic electron flow (CEF) systems, the NDH and PGR5/PGRL1, were downregulated (Fig. 4.8). CEF has been known for decades, however to date only the identity of the subunits and the overall structure is considered to be understood, however focus is being turned to its physiological role and evidence is starting to emerge that CEF is an important function in plants under low light and stress conditions (Suorsa et al. 2009; Suorsa 2015).

There are two modes of electron transport involving Photosystem I (PSI), one linear, through the photosystem on to the Calvin-Benson Cycle, the other cyclic, with electrons being routed back to the PQ pool (Fig. 4.6). This second process is termed cyclic electron flow, or CEF. Two complexes are known that perform CEF, and it is understood that they act separately, although under what conditions each operates is not known (Joliot & G. N. Johnson 2011; Suorsa 2015; Suorsa et al. 2016). It has been shown that both complexes accept electrons from ferredoxin (FD1) and pass them back to the plastoquinone (PQ) (Suorsa 2015; Yamori et al. 2011).

The NDH complex is a multiprotein complex comprising more than 30 subunits, that functions in CEF in the non-appressed thylakoid membranes of the chloroplast (Suorsa 2015). Fourteen genes encoding these complexes are regulated by BOLT. The identity of the genes that encode the proteins of the NDH, along with a number of ancillary proteins that assemble and stabilize the complex have only recently been established, and a model of the structure has been suggested (Fig 4.7) (Peng et al. 2011; Ifuku et al. 2011; Suorsa et al. 2009; Suorsa 2015). PGR5/PGRL1 also plays a role in CEF, and PGR5 is downregulated in our microarray.
Figure 4.10  BOLT overexpressing plants share phenotype with GDC bypass plants when both are grown in short (12-hour) days. A, model of a GDC with the bypass pathway shown in pink. B, wild type plants, left, GDC bypass mutant, right (Peterhansel et al. 2010). C, wild type, left, BOLT overexpressor, right.
As the aim of most of the research into the NDH complex has been to identify the subunits and structure, most of the work has been done under optimal growth conditions. The NDH comprises a significant number of proteins, so it could be expected that the complex would be critical to the survival of the plant, yet under usual growing conditions NDH-deficient plants appear normal (Shikanai 2007; Yamori et al. 2011; Yamori et al. 2015). Recently there has been some investigation into the status of the CEF systems under stressful conditions. An interesting result is that the abundance of the NDH complex has been shown to decline under various conditions such as cold, low light, drought, and heat, suggesting a role for the NDH complex in abiotic stress response or signaling (Yamori et al. 2011; Yamori et al. 2015; Ibáñez et al. 2010; García-Andrade et al. 2013; Ueda et al. 2012).

There is evidence that connects the phenotype we see in the BOLT overexpressing and RNAi plants with the inhibition of CEF. Plants that constitutively overexpress BOLT are much smaller in low light compared to wild-type plants, and the knockdown plants are somewhat larger (Fig 3.5). Yamori et al demonstrated that rice plants lacking the NDH complex grown under low light (50µM/m²s) or in cold temperature each had reduced plant growth with less biomass and lower grain yield than similarly treated wild-type plants (Yamori et al. 2011; Yamori et al. 2015). The plants grown at low light intensity also had lower measures of photosynthetic components, lower CO2 assimilation and lower electron transport rates than wild type (Yamori et al. 2015). Further evidence of CEF’s role in low light conditions is exemplified in Arabidopsis by three mutants lacking both CEF systems which were very small compared to wild type under low light conditions (Fig 4.11) (Munekage et al. 2004). The conclusion drawn by Yamori et al is
that NDH is not necessary for photosynthesis in normal light, but in low light it is important, and possibly has a dual role in maintaining photosynthesis in low light, while also acting as a safety valve to prevent over-reduction of the stroma under stress conditions (Yamori et al. 2011; Yamori et al. 2015). If CEF is required for photosynthesis in low light, it is reasonable that the RNAi plants would be larger than wild-type plants because express almost no BOLT which downregulates numerous CEF genes. This proposed dual role is interesting because of the phenotypes we see in the plants overexpressing BOLT. Under all conditions the plants flower early, and we propose this is a stress response, but under low light there is the additional phenotype of very small plants in the overexpressing lines and larger plants in the RNAi lines (Figs. 3.5).

Several additional papers further support the dual role concept for CEF. In Arabidopsis, pathogen attack destabilized the NDH complex, and in CEF impaired plants, disease resistance to fungal pathogens was substantially enhanced (García-Andrade et al. 2013). In Marchantia, an ndh mutant had a reduced PQ pool at low light intensity, and Ibanez et al showed that the NDH is the more important CEF complex in shade plants, while PGR5 is more important in sun plants lending more support to the notion that the NDH complex is important in low light (Ibáñez et al. 2010).

Ten of the CEF genes downregulated on our microarray experiment are also downregulated in the mkk1/2 double mutant, as well as in water deprivation, cold temperature, and flg22 treatments based on experiments collected at EBI’s Expression Atlas (Petrysak et al. 2013; Petrysak et al. 2016; Kapushesky et al. 2011). These data, which parallel the experimental data we show in Chapter 2, suggesting that the MAPK
Figure 4.11 In low light intensity CEF and BOLT mutants are small

A, cef mutants grown under low light (circled) (Yamori et al. 2015). B, BOLT overexpressing mutants (left and right) and wild type (center) grown under low light.
pathway may regulate CEF through BOLT in response to these stresses (Figs. 2.3 and 2.5). (Kapushesky et al. 2011; Petryszak et al. 2013; Petryszak et al. 2016).

Using the AGRIS AtcisDB database, I examined the promoter regions of the CEF genes that BOLT affects in the microarray, and found many DNA binding sites that are shared among the promoters of those genes (Palaniswamy et al. 2006; Yilmaz et al. 2010; Davuluri et al. 2003). None of the motifs are in all of the promoters however, suggesting a variety of transcription factors are probably involved in regulating the expression of these genes, but there is also the possibility that there is some common, but unknown motif(s) in the promoters. In our microarray, fourteen genes encoding CEF proteins were downregulated. This would be a large number of genes for BOLT to act on directly. The fact that FD1 is also downregulated could suggest this is the point at which BOLT activity affects CEF, and without electrons to accept, the complexes are not maintained.

Taken together, these data suggest that BOLT has a role in the response to and signaling of environmental stresses in Arabidopsis through its regulation of cyclic electron flow. Most of the work to date into CEF function has been under optimal growing conditions. Further investigation into BOLT’s effects on the CEF genes through the use of the RNAi lines and the constitutive overexpressors as well as environmental stress and light treatments will move the field of AP2/ERF transcription regulation forward as well as improve our understanding of the role of cyclic electron flow in biotic and abiotic stress signaling.

Both the photorespiration and cyclic electron flow systems are involved in abiotic stress, defense and light-related activities in party by controlling ROS levels. Both are
also negatively regulated by *BOLT*, which responds to stress and has a light-related phenotype in the overexpressor. These results taken together could suggest a role for BOLT in ROS regulation in response to abiotic and biotic stress, and insufficient light levels. Further investigation into the GDC, CEF, and BOLT mutants in response to pathogen, cold, and light, which has not yet been done, may produce additional interesting phenotypes which would demonstrate the effects of these systems in non-optimal conditions.

**BOLT may regulate defense response through photosynthesis and hormone signaling**

The next most enriched clusters have more terms and more genes than the clusters that contain only GDC or CEF genes (Table 2). A total of 44 genes are in Cluster 7 and 66 in Cluster 8, with a total of 96 unique genes between the two. Cluster 7 includes the GO terms for response to JA, auxin, GA, and SA. Cluster 8 includes JA and SA signaling terms, plus defense-related terms, “photosynthesis, light reaction”, “stromule”, and “MAPK cascade” (Table 2). Most of the terms represent sets of genes that are approximately 2 to 3-fold enriched compared to the genome as a whole. The photosynthesis and stromule terms are enriched 4 and 5-fold.

Cluster 7, comprises only “response to” hormone terms and interestingly, both the canonical defense-related hormones, SA and JA, as well as growth-related hormones, auxin and GA are included (Table 2). This suggests that there may be a role for BOLT in the balance plants must maintain between growth and defense. We see evidence of both in our expression results and in the early flowering phenotype (Figs. 2.3 and 3.5). We
know that *BOLT* expression decreases with exogenous application of SA or JA, that it acts downstream of an MAPK pathway that regulates SA, and that there is evidence it is regulated by genes involved in the biosynthesis of and response to SA, *PAD4*, *SID2*, and *NPR1* (Fig 2.3 and 2.5) (Brodersen et al. 2006; Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011). Together this evidence connects BOLT to SA and JA responses and signaling and suggests that further experiments testing *BOLT’s* expression in response to auxin and GA could be undertaken to elucidate their effects on *BOLT* expression. In addition, measuring the SA, JA, auxin, and GA content in the *BOLT* transgenic lines could indicate whether BOLT has a role in the biosynthesis or accumulation of these hormones.

Crosstalk between hormone pathways is a major research focus. There is now considerable evidence that GA and auxin, not traditionally linked to defense responses, have important regulatory roles in SA- and JA-mediated defense (Ballaré 2011; Erb et al. 2012; Robert-Seilaniantz & Grant 2011). One interesting non-result of the microarray is that although we had found in earlier experiments that BOLT expression increases in response to ABA, none of the differentially expressed genes cluster into ABA-related GO terms (Fig 2.3). This does not unequivocally show no role for BOLT in an ABA-related function, but does suggest that BOLT’s primary role in abiotic stress response could be in a ABA-independent pathway.

There is only one Cellular Component term in Cluster 8; that is “stromule” (Table 2). Stromules are stroma-filled tubular extensions formed by chloroplasts and other plastids whose functions are not yet understood, however it is suggested they may be a mechanism for communication with the nucleus and other organelles to coordinate
genetic programs and other cellular functions, although this has not been proven (Brunkard et al. 2015) (Köhler et al. 1997) (Hanson & Sattarzadeh 2011) (Schattat et al. 2012). Stromules have been shown to form in response to light-sensitive redox signals within chloroplasts, during plant immune responses, and in response to SA and H$_2$O$_2$, and they from dynamic connections with the nucleus during these responses (Brunkard et al. 2015; Caplan et al. 2015). This suggests BOLT could have a role in defense signaling between the chloroplast and the nucleus via the regulation of stromule formation.

The Biological Processes terms in Cluster 8 all point to a role for BOLT in defense response and defense signaling, but there is one abiotic stress term, “cold” that represents a significant number of genes (35) as well. Cold stress, as shown in our hypothetical model, as well as elsewhere in the literature, has pathways in common with other abiotic and biotic stress signaling (Fig. 2.4) (Solanke & Sharma 2008; Pitzschke et al. 2009; Furuya et al. 2014; Ichimura et al. 2006). In Chapter 2, I showed that BOLT expression increases substantially when plants are subjected to cold temperature, and it has been shown that the MAPK cascade including MKK1 and MKK2 also has a role in cold regulation with MKK2’s role being more prominent (Fig 2.3)(Teige et al. 2004). In addition, it has been shown that SA mediates response to cold temperature, dehydration, and salinity in addition to its well-known role in defense (Miura & Tada 2014). As discussed in the introduction, a number of AP2/ERF transcription factors have been shown to have roles in both cold temperature signaling and biotic stress, and cold is known to affect photosynthesis (Agarwal et al. 2006; Sakuma et al. 2002; Stitt & Hurry 2002). This suggests a role for BOLT in both cold and defense responses as well as the possibility of mediating crosstalk between the two.
Early flowering as a possible escape mechanism

According to Martinez et al, salicylic acid regulates flowering time and links defense responses to reproductive development (Martínez et al. 2004). The authors and others found that plants under stress can trigger the transition to flowering prematurely, and that accelerated flowering is dependent on SA accumulation with SA deficient mutants flowering late, and over-accumulating mutants showing early flowering transition (Martínez et al. 2004; Rivas-San Vicente & Plasencia 2011; Hara et al. 2007). It is also known that flowering transition is tightly regulated however various stresses including pathogen infection and extreme temperature can promote flowering (Raskin 1992; Xu et al. 2013).

There are a number of genes differentially expressed in the microarray that are implicated in circadian rhythm and transition to flowering. Two cyclic nucleotide-gated ion channels (CNGCs), CNGC4 and CNGC12, which are differentially expressed in the microarray have been shown to have roles both in flowering timing and defense (Fortuna et al. 2015). Nuclear Factor-Y (NF-Y) transcription factors are heterotrimeric complexes found in all higher eukaryotes. Three subunits, NF-YA4, NF-YA7, and NF-YB2 are differentially expressed in the microarray. NF-YA7 has been shown to have roles both in stress and in flowering time (Kumimoto et al. 2008; Wenkel et al. 2006), and NF-YB2 has been shown to also have a role in flowering time (Cai et al. 2007; Kumimoto et al. 2008). ZTL is the F-box component of an SCF complex implicated in circadian clock period, and LHY, and the related RVE8, both differentially expressed, both have been shown to have central roles in the circadian clock (Fogelmark & Troein 2014).

Although these genes have roles in circadian rhythm and transition to flowering,
they are not as readily classified as many of the genes we see being differentially expressed within the 24 hours after induction of BOLT overexpression. This is likely because flowering time is on an endogenous schedule and responds to environmental signals, but integrates them into the development program whereas defense genes are on more of a switch as they have to respond very quickly to changing conditions. Our microarray covered only a 24-hour period which may not have been sufficient to capture gene expression changes in clusters of related flowering time genes. Possibly had BOLT been overexpressed for a longer time period we would have started to see groups of genes responsible for the early flowering phenotype we see in the transgenic plants.

Methods

Plant growth

The wild-type Arabidopsis thaliana and the TRANSPLANTA Line, TPT_1.01250.1F used in this study are in the Col-0 background. The TRANSPLANTA line was purchased from the NASC. The plants were grown according to the Chapter 2 Methods.

Quantitative RT-PCR

qRT-PCR was performed according to Chapter 2 methods using the primers described for BOLT, PP2A, and UBI10.
**β-estradiol treatment and sampling**

Zero-hour samples (100mg, about 10 seedlings) of ten-day-old seedlings, wild type and inducible lines were taken before the rest of the samples were treated by spraying with 75µM β-estradiol in 0.15% EtOH. Samples were the collected after 8, 12, and 24 hours into microcentrifuge tubes and immediately frozen in liquid nitrogen. RNA was extracted and treated as described in Chapter 2 Methods and the quality assessed using a BioRad Experion automated electrophoresis system. Three micrograms per sample was preserved with RNA Stable (Sigma Aldrich 93221-001-1KT) for shipping to OakLabs, Hennigsdorf, Germany. One microgram per sample was reverse transcribed into cDNA and used in RT-qPCR both according to the procedures in the Chapter 2 Methods section, to confirm an increase in BOLT expression in the induced line.

**Microarray analysis**

Oaklabs, Hennigsdorf, Germany performed the microarray experiment using an Agilent platform and provided us the raw data. Our collaborators at Penn State, Dr. Réka Albert and Dr. Jorge G.T. Zañudo normalized the data using quantile normalization and performed a one-way ANOVA for each gene in each sample type. Using the Storey method, their statistical analysis generated sets of 208 and 1142 genes differentially expressed over the time course and between the samples \((q<.01 \text{ and } q<.05 \text{ respectively})\), between the wild-type and the induced transgenic line. Drs Albert and Zañudo also generated a co-expression network from the \(p<.01\) data set. A pair of genes is connected with an edge if the average time course has a Pearson correlation coefficient greater than 0.95 (positive regulation) or lower than -0.95 (negative regulation).
Chapter 5: Conclusions and Future Directions

*BOLT* regulates genes that affect defense and growth

In broad terms I have shown that BOLT, a putative AP2/ERF transcription factor, is expressed widely in Arabidopsis plants, localizes to the nucleus, and has an inhibitory role in both abiotic and biotic stress responses (Figs. 2.1, 2.2, ). *BOLT* responds to stress treatments and it acts downstream of a ROS-activated MAPK pathway that regulates SA- and JA- signaling and accumulation, and other abiotic and biotic stress responses (Figs. 2.3, 2.5, and 2.6) (Pitzschke et al. 2009; Qiu et al. 2008). The pathway has also been shown to negatively regulate stress responses (Kong et al. 2012).

The genome-wide expression analysis shows that *BOLT* affects the expression of numerous chloroplast- and plastid-related genes, and other genes that are involved in light-, hormone- and defense responses, mostly by inhibiting their expression (Table 2). BOLT also regulates genes encoding a key photorespiratory complex, the GDC, implicated in stress response, H$_2$O$_2$ generation, and photosynthesis, and genes encoding both cyclic electron flow systems, the NDH complex, and PGR5/PGRL1, which have an important role in photosynthesis especially under low light conditions (Table 2, Fig. 4.5 and 4.8).

These results move the field forward by identifying a role for the previously unpublished gene, by placing it in a known MAPK pathway, and by expanding that pathway to include discreet downstream systems. These results will serve to direct future research into this gene and possibly into other AP2/ERF or MAPK genes or further into the genetic relationship between photosynthesis and defense. Thus I have expanded my hypothesis to include our new findings (Fig. 5.1).
Abiotic stress responses (GDC, CEF)

SA, Pathogen

Cold, Salt, Dry

:BÖS:

MEKK1

MKK1

MKS1

SID2, NPR1

PAD4

Defense responses (GDC, CEF)

MPK4

LEC1, LEC2

ABI3, FUS3

Abiotic stress responses (GDC, CEF)

BOLT
**Figure 5.1 Expanded pathway model**

Proposed model including BOLT in the MAPK pathway. BOLT is also regulated by SA accumulation and signaling genes, PAD4, SID2, and NPR1, as well as by the embryogenesis regulators, LEC1, LEC2, FUS3, and ABI3. BOLT regulates downstream genes involved biotic and abiotic stress, including genes encoding complexes central to photorespiration and photosynthesis in stress conditions including low light.
Summary of findings

Originally hypothesized to be a guard cell specific transcription factor, using transgenic lines expressing 1666 bp of the native BOLT promoter driving a GUS reporter, I showed that BOLT is not specific to guard cells, but is expressed widely in the plant.

BOLT localizes to the nucleus and accumulates in nuclear bodies. I showed this shown using the 35S construct, which resulted in very bright, easy to identify bodies. To confirm that BOLT is not localized to the bodies for the purposes of degradation because it is overexpressed, transgenic lines using a native promoter could be generated and experiments performed as described below.

I tested BOLT’s expression in the mkk1/2 double mutant plants because in an analysis of BOLT expression in microarray and RNA-seq databases an experiment showed BOLT to be downregulated in this mutant. My experiments agreed. M KK1 and MKK2 are components of a known MAPK abiotic and biotic stress response pathway. I therefore hypothesized that BOLT regulates abiotic and possibly biotic stress response downstream from the MAPK pathway (Fig. 2.4). Further testing confirmed that BOLT responds to several types of abiotic and biotic stress.

Using transgenic overexpressing lines and amiRNA lines, I showed that overexpressing BOLT results in decidedly early-flowering, while knocking-down BOLT by >90% results in larger plants in low light and slightly earlier flowering. Thus, I propose that since BOLT responds to stress, the early-flowering is an escape measure due to an overwhelming stress response. The earlier-than-wild type flowering in the RNAi lines and the larger size in low light is related to CEF and the GDC being required in low light, not to the overexpressing stress response per se.
Expanding the model

I expanded our hypothetical model to include four SA-related genes that are regulated by MPK4 and that regulate BOLT. PAD4, SIZ1, SID2, and NPR1 have broad roles in SA-mediated defense responses (Jirage et al. 1999; J. B. Jin et al. 2008; Wildermuth et al. 2001; Dong 2004). Our microarray results showed numerous hormone response genes differentially expressed including those responding to SA, JA, GA, and auxin (Table 2). In addition, SIZ1 and NPR1 localize to nuclear bodies as BOLT may do as well (Saleh et al. 2016; Cheong et al. 2014). In order to more fully understand BOLT’s role concerning these hormones, I would measure the hormones’ levels under various conditions in the overexpressing lines and the amiRNA lines compared to wild type. The results could indicate if BOLT has a role in the increase or decrease of hormone levels and together with the microarray results, suggest experiments to further fill in the pathway that includes BOLT.

The GO terms analysis point further to a specific role for BOLT in abiotic stress response. Only one abiotic stress GO term, cold, is in any of the top eight annotation clusters (Table 2). Initially this was surprising, but it is suggested by the stress treatment results in which BOLT responded more strongly to cold than to other abiotic stresses (Fig 2.3). In addition, it is known that cold and defense responses are closely associated, affect photosynthesis, and that cold responses are mediated by the MAPK pathway that regulates BOLT (Miura & Tada 2014; Jeon & J. Kim 2013; Rasmussen et al. 2013; Qiu et al. 2008). It would be an important future line of inquiry to dissect the particular biotic
and abiotic stresses *BOLT* responds to and how BOLT transduces that information downstream.

Another unexpected finding is that *BOLT* expression is regulated in the knockout mutants of four transcription factors well known for their roles in embryogenesis (LEC1, LEC2, FUS3, and ABI4), and that *BOLT* regulates a significant number of genes that are also downstream from those genes according to our microarray results. This was surprising because although BOLT is expressed in the embryo, it is also elsewhere in the plant and our evidence points to a role in stress response (Figs. 2.1 and 2.3). This indicates that either BOLT has a separate role in embryogenesis or the embryogenesis genes have a role in stress response. The latter notion is somewhat supported by recent investigations (F. Wang & Perry 2013; Junker & Bäumlein 2012). To further examine the connection between these genes I would compare expression levels of *BOLT* and selected of the genes differentially expressed in the microarray that may also be regulated by one or more of the embryogenesis genes using lines defective in one or more of the embryogenesis genes. Most of the investigation into these embryogenesis genes has been done in seeds. This would be an opportunity to determine if they have a different or additional role in post-embryonic plants.

**Remaining questions**

There are, of course, many questions that arise from the results of any research. Various directions could be taken from genetics, to cell biology, to biochemistry. I am going to suggest a few avenues that I think are particularly interesting or important. Overall, ours is a genetics lab, and expanding the model we have proposed would be the
the likeliest course to follow, and I talk about that below. However, I will also mention a few other questions that arose as a result of the project that seem interesting, in part, because the results were unexpected.

35S::BOLT::YFP localizes to the nucleus in spots, suggesting nuclear bodies, or photobodies. These bodies were identified in the transient transformation of onion epidermis. Considering the evidence I have generated suggesting that BOLT is responsive to light, it is possible that BOLT is localizing to photobodies (Figs. 2.2 and 3.3 to 3.6). It is known that PHYB localizes to photobodies along with a number of PIF proteins and that BOLT is regulated in both the phyb and quintuple pif mutants (Leivar & Quail 2011; Leivar et al. 2008). In addition, it was shown that MPK4 and MKS1 form a complex with a stress-responsive WRKY transcription factor and that in addition to regulation by MKK1, MKK2, and MPK4, MKS1 also may regulate BOLT (Fiil & Petersen 2014; Petryszak et al. 2013; Petryszak et al. 2016; Kapushesky et al. 2011). We would be able to see if any of these proteins co-localize with BOLT by co-expressing fluorescently tagged versions along with BOLT in onion epidermis and visualizing the cells microscopically. If it appears as though any localize with BOLT, BiFC or FRET could be used to confirm an interaction. If we can determine proteins that co-localize or interact with BOLT in nuclear bodies, we would have information beyond the genetic pathway results, that could lead to an understanding of how BOLT carries out its role in abiotic and biotic stress response.

I tested BOLT expression in response to some abiotic and biotic stress treatments and can correlate those outcomes to the microarray results (Fig 2.3 and Table 2). However, the phenotypes I saw in the transgenic lines suggest a role for BOLT in
responding directly or indirectly to light, which is something I did not consider in our earlier stress-treatment experiments. BOLT has a number of light-related DNA-binding sites in its promoter (Table 1). The GDC and CEF genes that are differentially expressed in the microarray have been shown to respond to light, although it could be through ROS (Timm et al. 2015; Timm et al. 2012; Hoffmann et al. 2013; Suorsa 2015; Endo et al. 2008). Twenty-four genes with GO terms “response to light” (blue, red, and far-red) are regulated by BOLT in our microarray results (Table 2).

RNAi plants and plants overexpressing BOLT have light intensity-related phenotypes (Fig. 3.5 and 3.6). Prolonged activation of the MAPK cascade that results in BOLT regulation, leads to chloroplast damage in a light-dependent manner (Pitzschke et al. 2009). All of this suggests that it would be worthwhile to investigate how different light intensities affect BOLT and how BOLT affects downstream genes in different light conditions. Closely related to response to light is response to ROS. ROS in organisms, is delicately balanced based in part on light (Pitzschke et al. 2009). Since we have placed BOLT downstream of a MAPK pathway that is activate by ROS we should investigate whether ROS is accumulated in either the BOLT amiRNA or overexpressing plants.

**Filling in the pathway**

Those are a few specific questions suggested by the data, but the broader and, I think, more interesting question in a genetics lab is “What other genes make up the pathway?” A compelling question with any transcription factor is “what is its target(s)”.

This is interesting because we want to know the nitty-gritty of what goes on downstream where the real work is done. Our microarray experiment has resulted in an abundance of
very useful data that has resulted in a more complete understanding of BOLT’s role as a transcription factor as well as, along with our upstream, and phenotypic results, given us the basis to ask more specific question as to how BOLT’s regulation of downstream function changes the plant in response to changes in the environment.

To actually fill in the downstream pathway with BOLT’s immediate targets we could do a couple of different experiments. First, using our co-expression data, we could attempt to identify if any of the proteins whose genes are one or two edges away from BOLT are physical targets by doing binding assays with BOLT and possible binding sites we have identified using databases and the literature. This would be cumbersome, possibly unmanageable, and without a guarantee of any results. The second possibility would be to use ChIP-seq, which would return candidate target genes that could be tested, but at a very high cost in terms of funds and time.

Because Chip-seq is a prohibitively expensive experiment for many labs, downstream targets remain difficult to assess, and if ChIP-seq is performed, it is often limited to as few genotypes or conditions as possible. For those reasons, I think the next most efficient step in filling in the pathway would be to try to find out what’s upstream. What factors bind BOLT’s promoter either between the MAPK cascade and BOLT, or upstream from BOLT in another pathway. There is no evidence that BOLT is phosphorylated but it is a possibility so a first step might be to test if BOLT is a substrate for MPK4, MPK3, or MPK6, the latter two also implicated in biotic and abiotic stress response through the MEKK1 pathway (Blom et al. 1999; de Castro et al. 2006; Zulawski et al. 2013; Durek et al. 2010; Heazlewood et al. 2008). Although I wouldn’t rule direct phosphorylation out completely, from the results of various in silico analysis of BOLT’s
sequence, it seems more likely that BOLT is regulated by one or more transcription factor that are in turn activated or regulated by the MAPK pathway such as MKS1, an MPK4 substrate that has been shown to bind transcription factors in its stress response role (Fiil & Petersen 2014; Qiu et al. 2008; Andreasson et al. 2005).

To determine what transcription factors directly bind BOLT’s promoter, I would do a promoter pull-down assay. Briefly, in this assay, ~500nt overlapping fragments of BOLT’s promoter would be synthesized and biotinylated, incubated with protein extract, and separated using streptavidin-coated magnetic beads, and the resulting bound proteins identified using mass spectrometry. Resulting candidates could be confirmed using gel shift or $^{32}$P DNA-binding assays. The advantage of doing this type of experiment is that it is not cost-prohibitive and could be done using proteins from plants under a variety of stress conditions. This experiment could possibly confirm the model I propose that includes SA- and embryogenesis-related transcription factors as potential regulators of BOLT. Once transcription factors are confirmed, the assay could be used again to identify proteins that bind those transcription factors which could be very useful in filling in the pathway or pathways that include BOLT.

**Conclusion**

My research into BOLT serves as the first investigation into a gene that appears to have a very interesting role in the genetic integration of growth and defense. The results and analysis of this work have suggested a pathway and thus a framework for the further study of BOLT as well as the inquiry into two important systems, GDC and CEF, that are only recently understood to function in response to biotic and abiotic stress.
Appendix A

Fold-changes of the genes differentially expressed in the microarray experiment (q<.01) at 24 hours after induction of BOLT.

<table>
<thead>
<tr>
<th>Target Name</th>
<th>Gene Symbol</th>
<th>Description</th>
<th>24 hours Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G14150</td>
<td>CYP78A8</td>
<td>cytochrome P450, family 78, subfamily A, polypeptide 8 protein_coding</td>
<td>-40.65</td>
</tr>
<tr>
<td>AT1G17100</td>
<td></td>
<td>SGNH hydrolase-type esterase superfamily protein_coding</td>
<td>-31.83</td>
</tr>
<tr>
<td>AT1G32520</td>
<td>FAR5</td>
<td>fatty acid reductase 5 protein_coding</td>
<td>-25.50</td>
</tr>
<tr>
<td>AT1G48330</td>
<td>TBL19</td>
<td>TRICHOME BIREFRINGENCE-LIKE 19 protein_coding</td>
<td>-23.86</td>
</tr>
<tr>
<td>AT1G48750</td>
<td></td>
<td>Uncharacterised protein family (UPF0497) protein_coding</td>
<td>-16.03</td>
</tr>
<tr>
<td>AT1G49980</td>
<td>QRT1</td>
<td>Pectin lyase-like superfamily protein_coding</td>
<td>-15.14</td>
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<tr>
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<td></td>
<td>GDSL-like Lipase/Acylhydrolase superfamily protein_coding</td>
<td>-13.17</td>
</tr>
<tr>
<td>AT1G53270</td>
<td></td>
<td>pseudogene of unknown protein pseudogene</td>
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</tr>
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<td>scpl28</td>
<td>serine carboxypeptidase-like 28 protein_coding</td>
<td>-12.64</td>
</tr>
<tr>
<td>AT1G64780</td>
<td></td>
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</tr>
<tr>
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</tr>
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<td>PERK15</td>
<td>Protein kinase superfamily protein_coding</td>
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</tr>
<tr>
<td>AT2G42975</td>
<td></td>
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<td>AT5G02820</td>
<td>RXW8</td>
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<td>zinc finger protein 1</td>
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<td>unknown protein; BEST Arabidopsis thaliana protein match is: unknown protein</td>
<td>(TAIR:AT5G64190.1); Has 72 Blast hits to 72 proteins in 10 species: Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 72; Viruses - 0; Other Eukaryotes - 0 (source: NCBI BLink).</td>
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<td>CT-BMY chloroplast beta-amylase</td>
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<td>AT4G34630</td>
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<td>DURING: 13 growth stages; BEST Arabidopsis thaliana protein match is:</td>
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<td></td>
<td>Archae - 12; Bacteria - 1396; Metazoa - 17338; Fungi - 3422; Plants -</td>
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<td>5037; Viruses - 0; Other Eukaryotes - 2996 (source: NCBI BLink). protein</td>
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<td>Archae - 0; Bacteria - 0; Metazoa - 0; Fungi - 0; Plants - 74; Viruses - 0;</td>
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<td>Calcineurin-like metallo-phosphoesterase superfamily protein</td>
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Appendix A

This Appendix shows the fold-changes of the genes differentially expressed in the microarray experiment (q<.01) at 24 hours after induction of BOLT.

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