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Functional analysis of Arabidopsis cold shock domain proteins

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Functional Analysis of Arabidopsis Cold Shock Domain Proteins

Yongil Yang

Dissertation submitted to the Davis College of Agriculture, Forestry,
and Consumer Sciences at West Virginia University
in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
In
Genetics and Developmental Biology

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seed germination, plant development

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ABSTRACT

The cold shock domain (CSD) is one of the most evolutionarily conserved nucleic acid binding domains from prokaryotes to higher eukaryotes including plants. Although eukaryotic cold shock domain proteins have been extensively studied as transcriptional and post-transcriptional regulators during various developmental processes, an understanding of their functional roles in the Plant Kingdom is lacking. To understand the function of cold shock domain proteins *in planta*, I analyzed AtCSP3 (At2g17870), which is one of four *Arabidopsis thaliana* cold shock domain proteins (AtCSPs). Taq-Man probe quantitative RT-PCR (qRT-PCR) analysis confirmed that AtCSP3 transcripts were expressed dominantly in reproductive and meristematic tissues. The homozygous loss of function mutant *atcsp3* displays a distinct phenotype with an overall reduced sized of seedlings, small sized orbicular rosette leaves, and curled leaf blades. Microscopic visualization of cleared leaves revealed a reduction in size and increased circular shape of palisade mesophyll cells in *atcsp3* leaves. Image analysis of palisade cell layers indicated that the reduced size of the circular mesophyll cells is generated by a reduction of cell length and cell number along the leaf-length axis, resulting in an orbicular leaf shape. Also, I determined that leaf cell expansion is impaired for lateral leaf development in the *atcsp3* loss of function mutant, but the leaf cell proliferation is not affected. Loss of function of *AtCSP3* resulted in a dramatic reduction of *LNG1* transcript involved in two-dimensional leaf polarity regulation. Subcellular localization of AtCSP3 in onion epidermal cells revealed nucleocytoplasmic localization. Collectively, these data suggest that AtCSP3 regulates leaf length specific polarity by affecting *LNG1* transcript accumulation during leaf blade lateral expansion. I also discuss putative function of AtCSP3 as an RNA binding protein in relation to leaf development.

Eukaryotic cold shock domain proteins are nucleic acid binding proteins that are involved in transcription, translation via RNA chaperone activity, RNA editing, and DNA repair during tissue developmental processes and stress responses. Eukaryotic cold shock domain proteins have been functionally implicated in important developmental transitions, including embryogenesis in both animals and plants.

Arabidopsis thaliana Cold Shock Domain Protein 4 (AtCSP4; At2g21060) contains a well conserved cold shock domain (CSD) and glycine-rich motifs interspersed by two retroviral-like CCHC zinc fingers. GUS staining analysis in pAtCSP4:GUS transgenic Arabidopsis plants confirmed that *AtCSP4* was expressed in all tissues but accumulates in reproductive tissues and those undergoing cell divisions. Overexpression of *AtCSP4* resulted in a reduced length of siliques and embryo lethality. Interestingly, a T-DNA insertion *atcsp4* mutant did not exhibit any phenotypes, implicating that the similar *AtCSP2* gene is functionally redundant with *AtCSP4*. During silique development, overexpression of *AtCSP4* induced early browning and shrunken

seed formation beginning with the late heart embryo stage. A fifty percent segregation ratio of the defective seed phenotype was consistent with the phenotype of endosperm development gene mutants. Transcripts of *FUS3* and *LEC1* genes, which regulate early embryo formation, were not altered in the *AtCSP4* overexpression lines. On the other hand, transcripts of *MEA* and *FIS2* which are involved in endosperm development were affected by overexpression of *AtCSP4* indicating that *AtCSP4* may be a regulator of endosperm development via transcriptional or post-transcriptional regulation. Additionally, overexpression of *AtCSP4* also affected the mRNA generation of several MADS box genes in stages of early silique development. Specifically, transcripts of *AP*, *CAL*, *AG*, and *SHP2* were up-regulated. Collectively, these results indicate that *AtCSP4* plays an important role during the late stages of silique development by affecting the expression of several development related genes.

Cold shock domain proteins (CSPs) have been reported to play an important role in tissue development and cold responses. Eukaryotic CSPs play a crucial role in cell differentiation and cell proliferation which result in regulating the timing of tissue development and cell division. Cold shock domain proteins have been identified in many plants, although functional analyses have been limited to *Arabidopsis*, wheat and rice and their *in vivo* functional roles remain unclear. Among the four *Arabidopsis thaliana* CSPs (AtCSPs) that I have characterized, *AtCSP1* is highly similar to *AtCSP3* in terms of its predicted amino acid sequence. Transcription of *AtCSP1* is increased in the loss of function mutant *atcsp3*, implicating that *AtCSP3* negatively affects *AtCSP1* transcription. Using a gene trap line (GT606 defined as *atcsp1*), which has complete loss of full length *AtCSP1* transcript, GUS gene expression was detected preferentially in tissue primordia and highly dividing tissues. The *atcsp1* exhibited early germination after stratification but did not exhibit any further atypical phenotype in vegetative tissues. Germination of *atcsp1* without stratification also occurred earlier than wild type but the germination time delayed 24 hours. Comparative analysis of GUS expression in seeds with or without stratification confirmed that *AtCSP1* expression was affected by cold temperature during radicle emergence. In addition, ABA germination assays revealed a reduced sensitivity to ABA in *atcsp1*. Taken together, *AtCSP1* may function to regulate germination timing which is in turn mediated by cold temperatures to promote embryo expansion.

DEDICATION

This work is dedicated to
my wife, parent,
and all of the people who
have helped and supported me

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LITERATURE REVIEW

CSP structure

The structures of cold shock domains from bacteria, archaea, and human have been solved and have been reviewed in relation to their functions (Horn 2007, Giaquinto 2007, Kohno 2003¹⁾). The cold shock domain contains two well conserved RNA binding motifs which are denoted as RNP-1 and RNP-2 (Landsman 1992). The crystal structure of *Bacillus subtilis* CspB revealed that the CSD contains five β -barrel strands connected by a long flexible loop (Schindelin 1993). RNA binding motifs of RNP-1 (K/R-G-F/Y-G/A-F-V/I-X-F/Y) and RNP-2 (L/I-F/Y-V/I-G/K-N/G-L) reside in the second and third β -barrel strands (Graumann 1996, Schroder 1995, Landsman 1992). *E. coli* CspA also forms a similar structure with five anti-parallel β strands forming a closed five barrel structure containing several basic/aromatic islands that are rich in arginine and aromatic residues (Schindelin 1994). These basic/aromatic residues enable the CSD to bind preferentially to nucleic acid strands. Based on the high homology of the CSD in bacteria and eukaryotes, five β -barrel anti-parallel strands are regarded as the standard three dimensional structure for the CSD. Plant CSPs typically contain the conserved cold shock domain and auxiliary domain such as glycine rich regions interspersed by zinc finger motif (Thompson 2008, Karlson 2003). The CSD of YB-1 showed various specificities in the binding to RNA and DNA (Ladomery 1994). For example, it showed high affinity with polypurines poly (A, G) and poly G but not polyA in the presence of 5 mM MgCl₂. In 1 mM MgCl₂ or 1 mM spermidine, it interacts preferentially with poly (C, U) (Ladomery 1994). The nucleic acid binding activity of CSD is enhanced by the existence of C-terminal auxiliary domains (Nakaminami 2005, Ladomery 1994). The three dimensional structure of CspB showed that it has a suitable stereographic structure to bind to nucleic acid backbone, which induces Csp binding activity to nucleic acids (Skabkin 2004).

Bacterial cold shock domain protein

Cold shock domain proteins (Csp) were first reported in bacteria by Goldstein and his colleagues (Goldstein 1990). The *E. coli* Csp family contains nine different *csp* genes designated

¹⁾ All citations, including multi-authors citations, show only first author's name.

as *csp A* through *I* which differ widely in their cellular functions (Yamanaka 1998). Among the nine cold shock proteins, *cspA*, *cspB*, *cspG* and *cspI* (Wang 1999, Etchegaray 1996, Nakashima 1996, Lee 1994, Goldstein 1990) are reported to be involved in cold stress. On the other hand, *cspC* and *cspE* are constitutively expressed but function as multi-copy suppressors of a temperature-sensitive protein. The *cspD* gene is not induced under cold temperature but is highly expressed under nutritional deprivation and normal stationary growth stage in *E. coli* (Yamanaka 1997 and 1994). The functions of *cspF* and *cspH* are not clear in relation to cold shock and during stages of bacterial growth. The aquatic bacterium, *Caulobacter crescentus*, also contains four *csp* genes of which only *cspA* and *cspB* were induced by cold shock (Lang 2004). Therefore, *csp* proteins function in diverse cellular development and stress responses even though their nomenclature was first defined by a response to cold.

A cold sensitive bacterial mutant (BX04) was generated by a quadruple deletion of *cspA*, *cspB*, *cspG*, and *cspE* resulting in cold sensitivity and filamentous cellular growth (Xia 2001). With the exception of *cspD*, overexpression with other *csp*s complemented the cold sensitivity of this quadruple deletion strain. These data indicate that members within the *E. coli* cold shock protein family are functionally redundant.

Bacterial *Csps* bind single strand nucleic acid strands such as RNA and DNA and induce nucleic acid antitermination. This activity has been observed in different *csp*s such as *cspA*, *cspC* and *cspE* (Bae 2000). Individual cells overexpressing a single *cspE* mutant or double *cspC* and *cspE* mutants showed a loss of nucleic acid binding and melting activity which resulted in cold sensitivity of bacterial cells. These results implicate that the nucleic acid binding and melting activity play an important role in cold adaptation of bacteria via modification of DNA or RNA structure (Phadtare 2006). This research also revealed promoter-proximal sequences containing many genes that are regulated by *cspC* and *cspE* at physiological or cold shock temperatures. The 5' untranslated region (UTR) of *cspA* mRNA was recognized by RNA binding proteins to unfold the secondary structure of *cspA* mRNA, inducing its own translation (Yamanaka 1997). Bacterial *Csps* have been implicated to play roles in transcription, mRNA stability and translation under cold stress and stationary condition (reviews: Graumann 1998, Sommerville 1999). *CspA*, the major cold shock protein in *E. coli*, was demonstrated to function as an RNA chaperone due to its RNA binding activity to destabilize RNA secondary structures, allowing

release of secondary structures of RNA for efficient translation or post transcriptional regulation of mRNA at low temperatures (Jiang 1997).

Bacillus subtilis contains three *csp*s which are denoted as: *cspB*, *C* and *D*. A double deletion of *cspB* and *cspC* altered protein synthesis, induced cell lysis during the stationary growth phase and impaired the ability of bacteria to differentiate into endospores (Weber 2001). Interestingly, the complementation of translation initiation factor IF1 from *E. coli* in a *B. subtilis* *cspB* and *cspC* double deletion strain rescues the loss of function for both the growth and the sporulation, implicating a shared function of IF1 with *cspB* and *cspC*. Archaeal *csp*s have also been identified from psychrophilic and mesophilic *Euryarchaeota* which include *Methanogenium frigidum* and *crenarchaeota* (Saunders 2003, Giaquinto 2007). Archaeal *csp*s binds to single-strand RNA similar to the single stranded nucleic acid binding activity of *E. coli* *csp*s (Giaquinto 2007). The growth defective *E. coli* mutant (BX04) was rescued by complementation of individual *csp* homologs and structural analogs from three psychrophilic archaea (Giaquinto 2007).

Eukaryotic CSD proteins

In eukaryotes, many different organisms such as nematodes, sea squirts, birds, fruit flies, mice, fish, plants, and humans contain at least one copy of a cold shock domain protein except yeast (Falsone 2002, Zend-Ajusich 2002, Chang 1999, Nambier 1998, Wada 1998, Thieringer 1997, Kingsley 1994, Tafuri 1992). Eukaryotic cold shock domain proteins contain an N-terminal cold shock domain (CSD) and C-terminal region with various functional domains. Y-box binding protein (YB-1; dbp), Upstream of N-ras (Unr), LIN28, RBP16, WCSP, OsCSP, and AtCSP are examples of eukaryotic cold shock domain proteins that have been functionally characterized from diverse organisms (Chaikam 2008, Karlson 2003, Kohno 2003, Karlson 2002, Haymann 1999, Moss 1997, Kingsley 1994, Doniger 1992).

YB-1 protein

The YB-1 protein has been extensively studied from various eukaryotic organisms including human (review: Kohno 2003). YB-1 was first identified as a MHC class II promoter binding protein (Didier 1988) which binds to the 5'-CTGATTGG-3' *cis*-element sequence. When aligned to the cold shock domain of bacterial *Csp*s, YB-1 proteins exhibit more than 40%

identity to the bacterial sequences. Human YB-1 proteins, *dbpA*, *dbpB/YB-1*, and *dbpC/contrin* were cloned and functionally characterized (Tekur 1999, Kudo 1995). Immunofluorescent cellular localization studies confirmed that *dbpA* is localized both in the cytoplasm and the nucleus (Kudo 1995). The gene dispersion of *dbpA* and *dbpB/YB-1* within chromosomes is very different; *dbpA* is located at a unique position of chromosome 12. On the other hand, *dbpB* is dispersed on many chromosomes with multiple copies on chromosome 1. For this reason, *dbpA* and *dbpB* have been regarded as diverse functional genes even though they were derived from a common ancient origin. *dbpC/contrin* is expressed in human germ cells and is highly homologous to mouse *YB-2 (MSY2)* on a sequence level. *MSY2* is also highly expressed in germ cells (Gu 1998). *dbpC/contrin* is regarded as a potentially novel cancer/testis antigen because it is highly expressed in carcinomas but was restricted to germ cells and placental trophoblasts in normal cells (Kohno 2006).

YB-1 is a pleiotropic CSD protein in mammals which is involved in transcription, translation, mRNA editing, and DNA repair mediated by a base excision and mismatched DNA repair mechanism (Gaudreault 2004, Kohno 2003). YB-1 also plays a role in stress response and cell proliferation owing to its DNA repair ability. In general, YB-1 proteins are involved in transcriptional repression and activation which is mediated by its nucleic acid binding activity. YB-1 was first identified as a repressor of the gene expression of the human granulocyte-macrophage colony stimulating factor (GM-CSF) (Coles 2000, Coles 1996). The human VEGF promoter is also repressed by YB-1 which is phosphorylated by ERK2 and GSK3 β (Coles 2005). In addition, YB-1 mRNA transcription is regulated by p73 interacting with c-Myc in humans, which is a major functional gene of cell proliferation (Uramoto 2002). These results support that YB-1 proteins function as regulators of cell proliferation by transcriptional repression. However, in Jurket T cells, YB-1 functions as an activator of GM-CSF. In this case, the activation is not mediated by binding to single stranded DNA or RNA of GN-CSF. The activation is mediated by a protein-protein interaction at its C-terminal region with transcription factors such as RelA/ NF- κ B p65 (Diamond 2001). Similar to YB-1, *dbpA* and *dbpB/YB-1* have also been reported as activators of viral genes (Ansari 1999, Sawaya 1998, Swamynathan 1997, Raj 1996, Kerr 1994).

YB-1 readily forms a trimer, hexamer, and a 12 molecule oligomer in solution, but these structures revert to dimer conformation after the addition of ATP (Gaudreault 2004). The YB-1 dimer functions to separate the mismatched DNA complexes such as blunt ends, 5' or 3'

overhangs of double stranded DNA, and forked DNA structures (Gaudreault 2004). YB-1 possesses endonuclease activity which is mediated by binding to double stranded DNA and 3' to 5' exonuclease activity by its single strand DNA binding activity (Gaudreault 2004, Izumi 2001). These results confirmed that YB-1 is involved in DNA repair by the recognition and repair of mismatched or excised base pairs in DNA. Thus it is not surprising that YB-1 has been shown to play a role in repairing DNA damage resulting from UV irradiation (Koike 1997).

YB-1 autoregulates its own translation by sequence specific binding to the 3' UTR of its own mRNA (Skabkina 2005 and 2003). Thus, the binding of YB-1 to its own mRNA negatively regulates YB-1 protein translation (Skabkina 2005). This process is competed with poly (A)-binding protein (PABP), which activates YB-1 protein translation. FRGY2, a *Xenopus* cold shock domain protein, exhibits cytoplasmic RNA binding activity which mediates transcription repression via masking maternal mRNA. The masking of maternal mRNA results in the accumulation of mRNA storage particles in *Xenopus* oocytes (Matsumoto 2000 and 1996). YB-1 is a major part of cytoplasmic mRNPs which mediate protein translation (Skabkina 2004). YB-1 possesses sequence specific mRNA binding activity suggesting that YB-1 can recognize specific mRNA (Swamynathan 2000, Bouvet 1995). YB-1 activates or inhibits mRNA translation and this activity is dependent on the quantity of the target genes mRNA abundance. In a low ratio of YB-1 protein/mRNA of α -globin, a monomer of YB-1 binds to mRNA, generating a typical mRNP complex. However, YB-1 is associated with mRNA to make a multimer complex which induces untranslatable mRNPs (Skabkina 2004). Studies with FRGY2 showed that YB-1 competes with 5' cap binding protein and RNase to switch mRNA translation depending on the amount of YB-1 protein (Evdokimova 2001).

In another example linking YB-1 to protein translation, the iron-responsive element-binding protein (IRP-BP/IRP2) is a binding target of YB-1 which results in the positive regulation of ferritin protein translation (Ashizuka 2002). The normal secondary structure formation in 5' UTR of ferritin mRNA is prevented by IRP2 which inhibits ribosome binding for the initiation of translation under low iron condition. Under high iron condition, the binding of YB-1 to IRP2, which is mediated by iron, allows the release of the secondary structure of ferritin mRNA resulting in the initiation of ferritin protein translation.

During cell cycle progression for cell proliferation, YB-1 is trans-localized from the cytoplasm to the nucleus at the G1/S phase transition in dividing cells. On the other hand, YB-1

localizes in the cytoplasm during stationary cell growth conditions (Jurrchott 2003). The transcription of cyclin A and cyclin B1 are induced by the overexpression of YB-1, suggesting that YB-1 facilitates cell cycle transition for cell proliferation. The nuclear translocation of YB-1 is regulated by phosphorylation at Ser102 which is mediated by Akt kinase (Sutherland 2005). Disruption of phosphorylation causes an accumulation of YB-1 in the cytoplasm and not in the nucleus, resulting in an inhibition of tumor cell growth.

Lin 28

In the *C. elegans* model system, *Lin 28* plays a critical role in determination of developmental timing by influencing the translation or stability of specific mRNAs (Morita 2006). *Lin 28* contains an N-terminal CSD and CCHC retroviral-type zinc finger motifs. *Lin 28* was first identified as a regulator of the second larval stage in *C. elegans* (Moss 1997). *Lin 28* functions in post-transcriptional regulation and its gene expression is down-regulated by the complementary binding of *Lin-4* transcript to the 3' UTR of the *Lin 28* mRNA (Moss 1997). *Lin28* homologs were identified in *Drosophila*, *Xenopus*, mouse, and human (Guo 2006, Moss 2003). Micro RNA regulation via binding of *Lin4* and *Let 7* mRNAs to the 3'UTR of *Lin28* is well conserved in diverse animal (Moss 2003). *Lin 28* in mammalian cells localizes to diverse cell types of undifferentiated cells and associates with mRNAs. Under stress conditions, *Lin28* localizes to P bodies which are cytoplasmic processing bodies inducing RNA degradation and microRNA regulation (Balzer 2007). The CCHC zinc finger domain of *Lin 28* is very important to retain its localization to P-bodies.

RBP16

RBP16 was identified from the mitochondria of *Trypanosoma brucei*, a parasitic protozoan, and contains CSD at its N-terminus and a glycine and arginine rich C-terminal region resembling the RGG RNA-binding motif (Hayman 1999). The cold shock domain of RBP16 functions majorly for interactions with RNA or protein and the RGG-like motif enhances the nucleic acid binding activity of the cold shock domain (Miller 2003). *RBP16* functions in relation to insertion editing of CYb and A6 pre-mRNA *in vitro* (Miller 2006). This editing requires both the CSD and RRG-like motif to fully activate mRNA translation. Arginine-93 within the RGG-like motif on *RBP16* is methylated by protein arginine methyltransferases

(PRMTs). This methylation provides additional evidence that the function of cold shock domain proteins can be regulated on a postranslational level (Pelletier 2001 and 2000).

Unr

In mammals, the *Unr* gene was identified by a survey of amino acid sequences predicted from mammalian cDNA sequences (Doniger 1992). *Unr* consists of multiple repeats of cold shock domain regions. Unr interacts with ALL-1, which mediates chromosome translocations and fusion to partner genes in human acute leukemia (Leshkowitz 1996). Similar to other eukaryotic CSPs, *Unr* expression is regulated at the 5' and 3' UTR for its own translation (Dormoy-Raclet 2005). The *Unr* 3' UTR region specifically destabilized its mRNA structure and the 5' UTR showed internal ribosome entry site (IRES) activity which is negatively regulated by unr. These data demonstrate that *Unr* also autoregulates its protein translation via post-transcriptional regulation. Recently, the human rhinovirus-2 mRNA was reported as the target mRNA regulated by unr via RNA chaperone activity (Anderson 2007).

PIPPin

PIPPin, which is a rat brain enriched protein, contains two putative double stranded RNA-binding domains and a CSD (Nastasi 1999). PIPPin binds specifically to histone 1 and 3.3 transcripts and inhibits translation of these mRNAs (Raimondi 2003, Nastasi 2000). Interestingly, PIPPin is a target of sumoylation, which is a well-studied post-translational modification process (Bono 2007). Sumoylation is regarded as an important protein modification process for histones and the PIPPin complex.

Plant CSPs

Plant CSPs were first classified among glycine rich proteins (*GRPs*) by alignment of the conserved CSD sequence (Kingsley 1994). Until now, plant CSPs have been identified from single cell photosynthetic organisms to higher plants (Thompson 2008, Karlson 2003). A phylogenetic analysis categorized plant CSPs into two groups depending on the absence of presence of auxiliary C-terminal domains (Karlson 2003). Wheat CSP1 (*WCSP1*) is a cold-specific responsive protein which binds to double and single stranded DNA and RNA (Karlson 2002). This single stranded nucleic acid binding activity has also been demonstrated for

Arabidopsis, rice and moss CSPs (Chaikam 2008, Kim 2007). *WCSP1* exhibits mRNA anti-termination activity, implicating its function as an RNA chaperone for the destabilization of mRNA secondary structures (Nakaminami 2006). *WCSP1* and rice CSP (*OsCSP1*), and Arabidopsis CSPs *AtCSP2* (*AtGRP2/CSDP2;At4g38680*) and *AtCSP1*(*CSDP1;At4g36020*) rescued a cold sensitive quadruple Csp deletion bacterial mutant BX04 under cold conditions. These data confirmed that plant CSPs are capable of functioning similarly to their prokaryotic counterparts (Sasaki 2008, Chaikam 2008, Kim 2007).

AtCSP2 is functionally related to plant developmental processes and is also responsive to cold stress cold (Nakaminami 2009, Sasaki 2007, Fusaro 2007). RNAi transgenic *AtCSP2* plants exhibited two phenotypes of early flowering and less stamen number under normal growth conditions (Fusaro 2007). *AtCSP2* is the most abundant gene among the four AtCSPs and its mRNA abundance is the highest in flowers and siliques. These expression patterns mirror those of *AtCSP4* (*AtGRP2b;At2g21060*) (Nakaminami 2009). A comparative analysis of AtCSP expression in Landsberg (*Ler*) and Columbia (*Col*) ecotypes showed that *AtCSP4* expression is 1000-fold reduced in the *Ler* background relative to *Col*.

A CSP in the green algae *Chlamydomonas* (*NABI*) regulates light harvesting chlorophyll binding protein (*LHCBM*) mRNA which induces a component of light harvesting antenna. The regulation of *LHCBM* mRNA ultimately impacts the regulation of photosynthesis and thus this protein is of great importance (Mussnug 2005).

CHAPTER 1

Arabidopsis Cold Shock Domain Protein 3 (AtCSP3) regulates leaf length polarity cell expansion in lateral leaf

Abstract

The cold shock domain (CSD) is one of the most evolutionarily conserved nucleic acid binding domains from prokaryotes to higher eukaryotes including plants. Although eukaryotic cold shock domain proteins have been extensively studied as transcriptional and post-transcriptional regulators during various developmental processes, an understanding of their functional roles in the Plant Kingdom is lacking. To understand the function of cold shock domain proteins *in planta*, I analyzed AtCSP3 (At2g17870), which is one of four *Arabidopsis thaliana* cold shock domain proteins (AtCSPs). Taq-Man probe quantitative RT-PCR (qRT-PCR) analysis confirmed that AtCSP3 transcripts were expressed dominantly in reproductive and meristematic tissues. The homozygous loss of function mutant *atcsp3* displayed a distinct phenotype with an overall reduced sized of seedlings, stunted and orbicular rosette leaves, and curled leaf blades. Microscopic visualization of cleared leaves revealed a reduction in size and increased circular shape of palisade mesophyll cells in *atcsp3* leaves. Image analysis of palisade cell layers indicated that the reduced size of the circular mesophyll cells is generated by a reduction of cell length and cell number along the leaf-length axis, resulting in an orbicular leaf shape. Also, I determined that leaf cell expansion is impaired for lateral leaf development in the *atcsp3* loss of function mutant, but their leaf cell proliferation is not affected. Loss of function of *AtCSP3* resulted in a dramatic reduction of *LNG1* transcript involved in two-dimensional leaf polarity regulation. Subcellular localization of AtCSP3 in onion epidermal cells revealed nucleocytoplasmic localization. Collectively, these data suggest that AtCSP3 regulates leaf length specific polarity by affecting *LNG1* transcript accumulation during leaf blade lateral expansion. I also discuss a putative function of AtCSP3 as an RNA binding protein in relation to leaf development.

Introduction

The cold shock domain (CSD) is regarded as one of the most conserved protein domains that is widespread from bacteria to mammals. The CSD contains two consensus RNA binding motifs, defined as RNP1 and RNP2, which facilitate functions like nucleic acid binding, RNA chaperone activity, post transcriptional regulation, and transcription regulation (for review; Horn 2007, Sommerville 1999, Graumann 1998). As the name implies, not all cold shock proteins (CSPs) in an organism are functionally involved in the cold stress response. For example, only four out of the nine *E. coli* CSPs named CspA, CspB, CspG, and CspI, are induced in response to cold stress (Lee 1994, Nakashima 1996, Wang 1999). Other *E. coli* CSPs such as CspC, CspD, and CspE are not induced by cold stress and CspC and CspD are induced by nutrient stress, whereas CspE can be induced at normal physiological temperatures. (Yamanaka 1997 and 1994). In eukaryotes, cold shock domain proteins contain various N-terminal and C-terminal auxiliary domains in addition to the CSD. The well studied human cold shock domain protein (Y-box binding protein-1; YB-1), performs pleiotropic roles in gene transcriptional regulation, DNA repair, and external stimuli response such drug resistance (reviewed by Kohno 2003). YB-1 activation by Akt-mediated phosphorylation results in its translocation to the nucleus which leads to the regulation of cell proliferation in human ovarian cancer cells (Basaki 2007). In addition, phosphorylation of YB-1 mediated by Akt-1 is regarded as the inducer for increase in expression of genes related to cell proliferation and stress response by translation of silent mRNA (Evdokimova 2006).

Since Kingsley and Palis first description of plant CSPs (Kingsley 1994), functional studies have been initiated in rice, wheat and Arabidopsis. Phylogenetic analysis of plant CSPs confirmed that they are highly conserved in the Plant Kingdom. Two types of plant CSPs were suggested to exist based on the presence or absence of auxiliary domains (Karlson and Imai 2003). *In vitro* functions of a wheat CSP (WCSP1), two rice CSPs (OsCSP1, OsCSP2), and two Arabidopsis CSPs (AtGRP2; AtCSP2, AtCSDP1) were reported as nucleic acid binding proteins. These studies suggest a putative functional role as RNA chaperones, although their precise biological role *in planta* remains elusive (Chaikam 2008, Sakai 2007, Kim 2007, Fusaro 2007, Nakaminami 2006, Karlson 2002). The first functional analysis for an Arabidopsis CSP (*AtGRP2/CSDP2/AtCSP2*) by RNAi induced gene silencing resulted in early flowering, reduced stamen number and abnormalities during seed embryogenesis (Fusaro 2007). These observations

clearly indicate that *in planta* functions of AtCSPs are not restricted to abiotic stress and may have important functions in plant development. The relationship to plant development was further supported by a recent detailed investigation monitoring the entire *AtCSP* gene family throughout all stages of development (Nakaminami 2009). However, the precise *in planta* functions of AtCSPs are poorly understood at the molecular and functional level.

Plant leaves are the major organ which absorb light energy and convert it into a storable energy source. For this reason, leaf size and shape are important determinants for overall plant growth and development. Leaf organogenesis is divided into three stages of leaf initiation, leaf polarity establishment, and cell expansion which results in final leaf shape (for reviews: Tsukaya 2006 and 2005). In general, these three stages of leaf organogenesis are regulated by different gene groups (for reviews: Barkoulas 2007, Byrne 2005). Leaf shape is initially established at two parts, leaf petioles and leaf blades. Arabidopsis leaf blades expand in two-dimensional flat directions which are defined as longitudinal (leaf-length) and lateral (leaf-width) axes. The ratio of the two directions is used as the criterion to determine leaf blade shape, which is further determined by the cell distribution, cell size and their interaction in leaf lamina.

ROTUNDIFOLIA 3 (ROT3), which is a plant type cytochrome P450 (CYP) 90C1, and ROTUNDIFOLIA 4 (ROT4) are regulators of leaf length polarity growth. A null allele of *rot3* exhibits stunted leaf and floral growth. Overexpression of *ROT3* in the *rot3* mutant background complemented the stunted leaf phenotype (Kim 1998, Tsuge 1996). Overexpression of *ROT3* results in longer leaves in the longitudinal direction but is not altered in leaf width. In contrast to ROT3, CYP90D1, a regulator of Brassinolide (BR) biosynthesis, showed a dwarf phenotype only when a double mutant was created in combination with the *rot3* null allele background. An enhancer trap line of ROT4, whose protein encodes a membrane bound small peptide, possesses a similar leaf shape phenotype as that of *rot3*, but the anatomical observation demonstrated that the stunted and small leaf shape in *rot4* is caused by a decrease in cell proliferation unlike that of ROT3. Other leaf polarity regulation genes in the leaf-length direction, *LONGIFOLIA* (*LNG1*) and *LONGIFOLIA2* (*LNG2*), were reported recently and were shown to function independently of ROT3 (Lee 2006). An activation tagged line of *lng 1-ID* exhibited a long leaf blade phenotype along with serrated margins and other elongated tissues. *lng1* and *lng2* loss-of-function mutants were found to have characterized to have a shortened length of leaf blades.

In the case of leaf width polarity specific regulation, loss of function of *ANGUSTIFOLIA* (*AN*), *SPIKE1*, and *ANGUSTIFOLIA3* (*AN3*)/GRF-INTERACTING FACTOR1 (*AtGIF1*), and overexpression of *AtHB13* (*OxAtHB13*) caused a similar narrow leaf shape in the leaf-width direction (Kim 2002, Qiu 2002, Folker 2002). *AN* protein, which is similar to the members of the animal CtBP protein family, arranges cortical microtubules to allow polar expansion of leaf cells. *SPIKE1* plays a role in cytoskeletal reorganization, which determines overall cell shape and tissue development. Therefore, it was hypothesized that leaf-width polarity regulation is related to cytoskeleton formation which is comprised of cortical microtubules. A loss-of-function mutant of *AN3* showed a narrow leaf phenotype in the leaf-length direction which is caused by the defect of cell numbers in leaf blades (Kim 2004). *AN3*, a homolog of the human transcription factor SYT, accumulated in leaf primordia and regulates cell proliferation. *AN3* gene expression in leaves overlapped with other Arabidopsis growth regulator factors (*AtGRFs*), *AtGRF5* and *AtGRF9*, but not with *AtGRF8*. Also, yeast two hybrid analysis identified an interaction between *AN3/AtGIF1* and *AtGRF5* and *AtGRF9* (Kim 2004). Overexpression of *AN3* and *AtGRF5* showed a 20-30% larger leaf size compared to the wild type, which resulted from an increase in leaf cell number (Kim 2004).

By characterizing two independent T-DNA insertion alleles, which exhibited small and stunted leaf shape, we report the first direct evidence of *AtCSP3* functioning as a regulatory protein in plant growth and development. In addition, we report two genes that are affected by *AtCSP3* during leaf development in Arabidopsis.

Materials and Methods

Plant material and culture conditions

Seeds of *atcsp3-1*, *atcsp3-2*, and *atcsp3-3* T-DNA insertion mutants were obtained from the Arabidopsis Biological Research Center (ABRC) with stock numbers of SALK_144972, Wisc_DsLox353G12, and SALK_022658, respectively. Col-0 wild type seeds were purchased from Lehle Seeds (Round Rock, TX). Prior to planting, seeds were stratified for 4 days at 4 °C without light. All plants were grown in Metromix 360 (Scotts Co., Marysville, OH, USA) under 16 h/ 8 hr and 8 hr/ 16hr of light/dark for long day and short day conditions, respectively, at 23 °C.

Measurement of gene transcript abundance

For measuring *AtCSP3* transcript in different tissues, total RNAs, with the exception of leaf tissues, were extracted with Plant RNA extraction Reagent (Carlsbad, CA, Invitrogen). Total RNA from rosette and cauline leaves were extracted with TRIzol® reagent (Carlsbad, CA, Invitrogen). First strand cDNA was synthesized with 500 ng of total RNA by the QuantiTect Reverse Transcription kit (Germantown, MD, Qiagen). Detailed protocols for RNA extraction and cDNA synthesis were obtained from the manufacturers. The generated cDNA was diluted fifty-fold for qRT-PCR and five-fold for semi-quantitative RT-PCR, respectively.

Root tips and elongation areas were separated by cutting roots from 10 day-old seedlings at 3 mm above the root tip. To obtain RNA from root maturation area, root tissues between 3 mm and 1 cm from the root tip were used. For the collection of root tissue, Arabidopsis seedlings were grown vertically on 0.5 X MS/1 % agarose plates including 1 % sucrose at 10 day after germination (DAG). Rosette leaves and shoot apical meristems were collected from 21 DAG plants grown in soil under the same light and temperature conditions as described above. RNA from cauline leaves and reproductive tissues were extracted from 28 DAG and 35 DAG plants, respectively.

TaqMan Probe qRT-PCR was performed with TaqMan® Universal PCR Master Mix (Foster City, CA, Applied Biosystems) with thermo cycling condition as follows: 95 °C for 10 min followed by 50 cycles of 95 °C for 15 sec and 60 °C for 1 min. Taq-Man probes for *AtCSP3* and *actin 2* were prepared from Applied Biosystems and their respective sequences are listed in Table 1-3.

For the confirmation of *atcsp3* T-DNA insertion alleles, cDNA was synthesized with total RNA extracted from leaf tissue of 28 DAG wild type Col-0 and loss of function mutant alleles. cDNA synthesis was performed as described above. Primer sequences for genotyping analysis are described in Table 1-2. PCR conditions were set up as follows: 95 °C for 2 min for initial denaturation, followed by 30 cycles of 95 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec, and a final extension for 7 min at 72 °C. Arabidopsis *actin 1* (*AAC1*) was amplified as an internal control for 26 cycles with the same PCR conditions as described above.

For semi-quantitative RT-PCR analysis of leaf cell polarity elongation genes, total RNA was extracted from the 5th leaf from three different soil grown plants under long day conditions for 28 DAG. Primers for *CYCD3;1*, *ROT3*, *LNG1*, *LNG2*, *AN3*, and *AN* were designed by

Primer3 software using the cDNA sequence information obtained from the Arabidopsis Information Resource (www.arabidopsis.org). PCR reactions were performed with a Go-Taq Flexi PCR reaction kit (Madison, WI, Promega). Thermal cycling conditions were set up as follows: 95 °C for 2 min for initial denaturation, followed by 30 cycles of 95 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec, and a final extension of 7 min at 72 °C. *AAC1* was used as an internal control amplified by the same PCR conditions as above.

Morphological analysis of loss of function mutant of AtCSP3

Wild type Col-0, *atcsp3-2*, and *atcsp3-1* were grown under long day conditions at 23 °C up to 56 DAG for morphological analyses. Representative photographs were taken a plant among 20 different plants. For root elongation and germination tests, sterilized seeds were grown on 1X Murashige and Skoog (MS) + vitamin B5 mixture / 1 % sucrose/ 1 % phytoagar plates under long day conditions. For assessment of root elongation, seedlings were grown vertically for 5 DAG on 1X MS/1 % agarose including 1 % sucrose, and 5 DAG plants which had the same root length were transplanted to new plates and maintained under the long day conditions. Root elongation was determined by measuring the difference between root length at 4 days after transplanting and initial root length at the time of transplanting.

Whole plants, aligned siliques, and leaf photographs were taken by a Nikon Coolpix 8700 digital camera (Melville, NY, Nikon). Additional photographs of small sized tissues such as flowers and seeds were taken under a Nikon SMZ-U dissecting microscope equipped with a Nikon DXM 1200 CCD camera (Melville, NY, Nikon).

Microscope observation and anatomic analysis

For observation of palisade cells, leaves were fixed in Farmer's fixative (ethanol: acetic acid 3:1) for 2-4 hours. Chlorophyll was completely removed by washing with 70 % and 100 % ethanol. To clear leaf tissue for microscopic observation, fixed leaves were soaked in 5 N NaOH at 60 °C for 2 hours. Nomarsky images were taken with a Nikon ECLIPSE E600 differential interference contrast (DIC) microscope equipped with a Nikon DXM 1200 CCD camera system (Melville, NY, Nikon). To observe epidermal cells on adaxial leaf surfaces, live 5th leaf tissue from a 28 DAG plant was stained with 100 µg/ml propidium iodide diluted in 0.1 M L-arginine buffer (pH 12.4) for 2-5 min. Stained leaf pictures were taken by a Zeiss Axioimager LSM 510

confocal microscope with Z-stack image generation mode. Dark field microscopic images were taken by a Nikon ECLIPSE TE2000-S microscope (Melville, NY, Nikon).

Subcellular localization by DNA coated particle bombardment

The coding region for the *AtCSP3* gene was amplified with primers containing the following restriction enzyme digestion sites; 5'-TCTGTCGACATGGCGATGGAAGATCAATC-3' and 5'-AGACCATGGTTTAAGCAACCGAAGTACATT-3'. Amplified PCR products were purified and digested with *NcoI* and *SalI* and inserted into a pre-digested sGFP(s65T) vector. DM-10 tungsten particles (Hercules, CA, BIO-RAD) were coated with 1 µg of plasmid. Particle bombardment was carried out into onion epidermal cells by using a Biolistic® PDS-1000 particle bombardment system (Hercules, CA, BIO-RAD) using the following conditions: 25 inches of Hg vacuum, 1000 psi rupture disk, and 12 cm target distance. Bombarded tissue was incubated at 20 °C for overnight in the dark. Images were obtained by a Zeiss Axioimager LSM-501 confocal microscope and analyzed by LSM image analysis software (German, Carl Zeiss AG).

Results

***AtCSP3* gene structure and tissue specific accumulation of mRNA**

AtCSP3 (At2g17870) is one of four *Arabidopsis thaliana* cold shock domain proteins (AtCSPs). The genomic sequence of *AtCSP3* consists of a single exon without any introns with a complete open reading frame of 906 base pairs, encoding a protein of 301 amino acids (Figure 1-1A). The *AtCSP3* protein consists of a well conserved N-terminal cold shock domain (CSD) and seven C-terminal CCHC retroviral-like zinc finger motifs that are interspersed by glycine rich regions. Quantitative real-time PCR (qRT-PCT) analysis was used with gene specific Taq-Man probes to characterize tissue specific mRNA accumulation patterns for *AtCSP3*. qRT-PCR results indicated that *AtCSP3* transcripts were accumulated in all vegetative and reproductive tissues (Figure 1-1B). However, *AtCSP3* transcripts were enriched in reproductive tissues and tissues with active cell divisions. *AtCSP3* transcripts were differentially expressed in different root regions. In root tissue, *AtCSP3* transcripts were highly accumulated in the maturation area

compared to the elongation zone and root tips. In aerial tissues, *AtCSP3* transcripts were detected highly in tissues with active growth and cell divisions like shoot apex, inflorescence, and siliques. Relative mRNA accumulation levels for *AtCSP3* were lower in leaves and dry seeds. Clear differences in *AtCSP3* transcript accumulation were observed between siliques containing full mature seeds before drying and seeds that were completely dried, indicating that *AtCSP3* gene transcription decreases during the seed dormancy process. Taken together, qRT-PCR results indicated that the *AtCSP3* gene is expressed in many tissues but shows elevated expression in meristematic and actively dividing tissues.

Molecular analysis to isolate *atcsp3* mutant alleles

We checked the SIGnAL T-DNA Express web-site to identify all available mutant lines containing putative T-DNA insertions in the *AtCSP3* locus (<http://signal.salk.edu/cgi-bin/tdnaexpress>). Two SALK lines (SALK_144972C, and SALK_022658C) and a Wisconsin line (WISC DsLox353G12) were identified and predicted to contain putative T-DNA insertions in the open reading frame or putative promoter region of *AtCSP3*. All mutant seeds were obtained from the ABRC stock center. Homozygous lines for each accession were confirmed by genotyping individual plants for several generations (data not shown). Sequence analysis of cloned PCR products with gene and T-DNA border specific primers was used to confirm the exact location of T-DNA insertions in each seed line. DNA sequence analysis confirmed that SALK_022658C has a T-DNA insertion in the promoter region far upstream from the initial codon of *AtCSP3* (Figure 1-2A, genotyping results are not shown). However, SALK_144972C and Wisc DsLox 353G12 have insertions at -42 into the 5'UTR and +170 in the coding region, respectively. WiscDsLox353G12 is denoted as *atcsp3-1*, SALK_144972C as *atcsp3-2*, and SALK_022658C as *atcsp3-3*.

To determine if *AtCSP3* gene transcript is knocked out in the T-DNA insertion mutants, we performed semi-quantitative RT-PCR using T-DNA left border (LB) and several gene specific primers designed at different positions in the sequences (Figure 1-2A). First, we performed PCR with non-flanking 3' UTR region primers (F1 and R1) to determine if *AtCSP3* transcripts were accumulated in the mutants. *AtCSP3* transcripts were detected in all T-DNA insertion lines with this primer set (F1+R1; Figure 1-2B). Thus, none of the *atcsp3* mutant lines are null mutants. We performed semi-quantitative RT-PCR with a forward primer flanking the

T-DNA insertion areas of *atcsp3-2* and *atcsp3-1* and the reverse R1 primer (Figure 1-1A). When F2 and R1 primers were used in *atcsp3-2* plants, a PCR product was obtained. However, when primers F3 and R1 primer (Figure 1-2B; F3+R1) were used, no product was obtained, indicating that these plants contain truncated *AtCSP3* transcripts. Similar analyses for *atcsp3-1* plants indicated that they also encode truncated transcript. When the multiple sets of primers were used with *atcsp3-3* cDNA, PCR products were identical to those of the wild type. Therefore, the T-DNA insertion in the *AtCSP3* promoter region in the *atcsp3-3* allele did not appear to affect *AtCSP3* gene transcription. Thus, it is unlikely that this mutation affected the function of the *AtCSP3* protein. Conversely, the *atcsp3-1* and *atcsp3-2* alleles are loss of function mutants generating 5' truncated *AtCSP3* transcripts and consequently alter *AtCSP3* protein function.

Phenotypic analysis of *atcsp3* T-DNA insertion mutant alleles

The function of *AtGRP2/CSDP2/AtCSP2* (At4g38680) was recently characterized by employing RNA interference (Fusaro 2007). Abnormal phenotypes such as early flowering, altered stamen number and abnormal seed development were observed for an RNAi line of *AtGRP2/CSDP2/AtCSP2*. Both *AtGRP2/CSDP2/AtCSP2* and *AtCSP3* contain an N-terminal cold shock domain, however, *AtCSP3* contains 5 additional C-terminal CCHC zinc finger motifs that are interspersed with glycine rich regions.

To observe the effects of the truncation in the *AtCSP3* transcript, we monitored the phenotype of T-DNA insertion mutants from germination through flowering and seed maturation. Under long day conditions, none of the mutants showed germination defects as determined by uniform emergence of radicles (data not shown). Primary root elongation was also measured on MS plate under long day conditions and no differences were observed among the mutant alleles (Figure 1-3 I).

During later stages of vegetative development, *atcsp3-1* and *atcsp3-2* mutants exhibited smaller and reduced number of leaves relative to wild type. However, *atcsp3-3*, whose transcript was not repressed or truncated, exhibited a phenotype identical to wild type plants (Figure 1-3A). Total leaf number from 28 DAG plants was compared with all alleles by counting all leaves after removing the primary stalk. Basal rosette leaf number of *atcsp3-2* and *atcsp3-1* was identical to the wild type. However, when the total leaf number counted including small younger leaves close to the shoot apex, the mutants had fewer total leaves than wild type (Figure 1-3B and G). In

addition to a reduced total leaf number, we also observed abnormally rounded leaf blades and short petioles in the *atcsp3-2* and *atcsp3-1* mutants. In contrast, *atcsp3-3* leaves had an identical shape to that of wild type plants. In addition to the aforementioned abnormalities in leaf shape, *atcsp3-1* leaves exhibited a curly phenotype. Curled leaves were not observed in the 1st and 2nd leaf pairs, whose leaf expansion started from 7 DAG, but very severe curling was observed in the 3rd leaves. *atcsp3-1* was the only allele exhibiting the severely curled leaf phenotype.

To determine if the mutant alleles showed any defect in the transition from vegetative to reproductive growth, we investigated flowering time by counting the days until the emergence of the primary inflorescence (~ 1 cm in height). Seedlings of the wild type and *atcsp3* mutant alleles started to flower approximately 22 DAG and were similar to the wild type (Table 1-1). When flowering time was investigated using short day conditions (6 hr/18hr for light/dark), no significant alterations in flowering time were observed (data not shown). Figure 1-3H shows that the height of full grown *atcsp3-2* and *atcsp3-1* plants are approximately 7 cm shorter than the wild type (Table 1-1). In addition, stem of *atcsp3-1* plant is thinner than the wild type at 28 DAG but *atcsp3-2* is not altered (data not shown). Measurements of fresh weight at 6 week DAG also indicated that growth of *atcsp3-2* and *atcsp3-1* plants is clearly reduced relative to wild type and *atcsp3-3*.

Among the reproductive tissues, *atcsp3-1* and *atcsp3-2* flowers were slightly shorter in length relative to the wild type. However, no abnormalities in floral organ shapes were observed in any alleles (Figure 1-3C). Regarding numbers of floral organ tissues, all alleles contained 6 stamens, 1 carpel, 4 petals, and 4 sepals (Figure 1-3D). Although the length of full-grown siliques of *atcsp3-1* and *atcsp3-2* were shorter than that of the wild type (Figure 1-3E), seed size was not altered in *atcsp3-1* and *atcsp3-2* mutant alleles. However, both mutants contained fewer seeds compared to the wild type and *atcsp3-3* (Figure 1-3, F and K). This effect was more pronounced in *atcsp3-1* relative to *atcsp3-2*.

In summary, morphological analyses of *atcsp3* T-DNA insertion alleles revealed that *atcsp3-2* and *atcsp3-1* mutants produce smaller plants with rounded leaf blades and shortened petioles. These same mutants also possessed shortened siliques and fewer seeds. Relative to other alleles and wild type, *atcsp3-1* exhibited severely curled leaf blades and a significant reduction in stem thickness. Consistent with RT-PCR data, which did not show a reduction in *AtCSP3* transcript (Figure 1-2), the *atcsp3-3* allele did not exhibit atypical phenotypes.

Leaf index measurement and orbicular leaf shape of *atcsp3*s

As shown in Figure 1-3, the stunted and orbicular leaf shape is the obvious phenotype in *atcsp3* loss of function mutants. To characterize the leaf shape of *atcsp3* T-DNA insertion mutants, I measured the length and width of the 5th leaf from 28 DAG seedlings. In wild-type plants, the shape of the 5th leaf is generally elliptical combined by the great length and short width of leaf blade. Wild type plants also have long petioles (Figure 1-4A). Conversely, leaf shape of the *atcsp3-2* and *atcsp3-1* mutants was rounded and orbicular with a pronounced reduction in petiole length. *atcsp3-1*, which contains a T-DNA insertion within the cold shock domain coding region, has a more severely stunted and rounded leaf phenotype which is accompanied by curling of the leaf surface. This phenotype was more severe than the one observed for the *atcsp3-2* allele. Both *atcsp3-1* and *atcsp3-2* alleles had reductions in the length of leaf blades and petioles and their plant sizes were significantly smaller relative to the wild type (Figure 1-4B). In contrast, leaf blade width was not as dramatically reduced as leaf length relative to wild type plants. To better characterize leaf shape, I calculated leaf index by measuring the length and width of the leaf blade area (Figure 1-4 C). Wild type Col-0 and *atcsp3-3* plants had 1.52 ± 0.11 and 1.60 ± 0.09 leaf index values, respectively. In the *atcsp3-2* and *atcsp3-1* mutant alleles, the leaf index values decreased to 1.33 ± 0.09 and 1.30 ± 0.10 , respectively. This reduction in leaf index values directly correlates to the increased roundness in leaf shape for these mutant alleles. In addition, leaf bases at the petiole junction were more rounded in *atcsp3-2* and *atcsp3-1* mutants.

Taken together, *atcsp3-2* and *atcsp3-1* leaves were orbicular in shape which resulted from a reduction of leaf length and a rounded leaf base. These mutations were also accompanied by shortened petioles. Since the *atcsp3-3* mutation did not result in reduction of transcript of *AtCSP3*, I eliminated *atcsp3-3* from further characterization due to its resemblance to the wild type.

Histological characterization of *atcsp3* mutants

To understand why the shape of leaf blades is altered in *atcsp3* mutants, we observed the size and shape of cells from epidermal and palisade mesophyll cell layers in the middle portion of 5th leaves 28 DAG. The most important difference observed among the wild type and mutant plants was the difference in cell size and shape (Figures 1-5A, B, and C). Adaxial epidermal cells

in *atcsp3-2* and *atcsp3-1* mutants were more densely arranged, while those of Col-0 wild type were more evenly aligned. The distribution of epidermal cells on the leaf surface of *atcsp3-1* mutants was uneven and condensed, which likely results in the curled leaf phenotype. Similarly, we also observed differences in cell distribution and size in palisade mesophyll tissue (Figure 1-5D, E, and F). The size of palisade cells in *atcsp3* mutants was smaller than that of the wild type. Similar to epidermal cells, the distribution of palisade cells in *atcsp3* mutants was more condensed relative to the wild type. In the *atcsp3-2* mutant, the palisade cells were more circular in shape compared to the wild type. This phenomenon was also observed to a lesser degree in the *atcsp3-1* mutant.

To understand why *atcsp3* mutants have orbicularly shaped leaves, I measured cell number and cell size on the same palisade cell layers. Cell length in both directions was counted from microscopic images of palisade cells from 5th leaf blades (Figure 1-5G and H). In microscopic images, the palisade cell layer from *atcsp3* appeared to contain more cells than the wild type. Since the leaf size of *atcsp3-1* and *atcsp3-2* mutants is smaller than that of the wild type, it is difficult to estimate the total number of cells in the leaf blade based on microscopic images within sections of leaves. Therefore, cell number in the microscopic image was multiplied with the ratio between the size of microscopic image and size of the original leaf to determine the total cell number. The estimated total cell number was similar between *atcsp3* mutants and the wild type (Figure 1-5G). Thus, I hypothesized that the reduced leaf size in *atcsp3* mutants might have resulted from alteration in cell number along the leaf-length or leaf-width direction. Counting cell numbers along both directions revealed that *atcsp3* mutants contain fewer cells along the leaf-length direction compared to the wild type. (Figure 1-5G). This alteration in the arrangement of palisade cells along leaf-length direction likely contributes to the formation of orbicular leaves in *atcsp3* mutants.

I also performed a comparative analysis to determine if the individual shape and size of epidermal cells is also altered in the mutants and correlated with the orbicular leaf shape mutation. To distinguish epidermal cells growing in leaf-length axis from cells growing in the leaf-width axis, I labeled cells “ α ” for leaf-length and “ β ” for leaf-width axes, respectively. On adaxial leaf surfaces, each epidermal cell and shape was observed and α and β shaped cells were evenly distributed in the epidermal cell layer of the wild type (Figure 1-5A). In contrast, comparative analysis of *atcsp3* loss of function mutants revealed a similar distribution pattern of

“ β ” shaped cells and a difference in the distribution of “ α ” shaped cells which grows majorly for leaf- length (Figure 1-5B and C).

I also counted cell number along leaf-length and leaf-width axes to understand the correlation between cell number along both directions and leaf shape. Using this method of characterization, wild type leaves have a greater distribution of cell numbers along the leaf-length axis than the leaf-width direction (Figure 1-5H). Thus, this cell distribution results in the typical elliptical shape of wild type leaves. In contrast, more palisade cells in *atcsp3-2* and *atcsp3-1* mutants are distributed in the leaf-width direction with less cells oriented in the leaf-length direction than that of the wild type (Figure 1-5H). Cell numbers along the leaf-width and leaf-length axes are nearly equivalent in the mutants resulting in the formation of their orbicular leaf blade shape.

To determine individual cell size, I measured the length along two-dimensions for epidermal and palisade cells. Cell length in both directions was determined by drawing a line that passed through the middle point of an individual cell in the leaf-length or leaf-width direction. ImageJ software was used to calculate cell lengths. Interestingly, cell lengths along the leaf-length axis on both cell types was significantly reduced in *atcsp3-1* and *atcsp3-2* mutants, whereas cell length along the leaf-width axis was not altered (Figure 1-5 I and J). These data indicate that leaf epidermal and palisade cells in *atcsp3-1* and *atcsp3-2* mutants are impaired in their expansion along the leaf-length axis but are similar to wild type in the leaf-width direction. Taken together, the results of cell dimension analyses indicate that the orbicular leaf shape of *atcsp3-2* and *atcsp3-1* results from impairment of the leaf cell polarity specific expansion in the leaf-length direction which is accompanied by an alteration of individual cell shape. In addition, the increase of cell size in the leaf-width direction is likely an additional contributing factor towards the development of an orbicular leaf phenotype.

To determine if the alterations in cell shape and size were responsible for generating the curly leaf blade phenotype in *atcsp3-1* mutant plants, we observed the curled areas with the same aforementioned approaches. In curly leaf areas, cell sizes in *atcsp3-1* mutants are reduced in both layers, with circular cell shapes in the palisade cell layer and shortened growth along leaf-width direction in epidermal cells (Figure 1-5C, F, I, and H). In general, the curly leaf phenotype initiates from 14 DAG, at which the 3rd and 4th leaves are expanding. 1st and 2nd leaves do not show a curled phenotype during leaf growth cycle (Figure 1-9A and B). Orbicular leaves tended

to be severely curved in the middle portions of leaf blades. Microscopic analysis of epidermal cells revealed a concentration of irregularly sized epidermal cells in curled areas, while more regularly sized epidermal cells were distributed in flat areas of leaves (Figure 1-9C). These observations indicated that the curly leaf phenotype of the *atcsp3-1* mutant is generated by irregularly sized cells distributed within curled areas of leaves.

***AtCSP3* is required for leaf cell expansion**

After cell determination in the shoot apical meristem, leaf morphogenesis proceeds in two separate stages including a cell division phase and cell elongation phase. Generally, cell proliferation by cell division occurs during early leaf development, whereas cell expansion continues until leaves mature into full size. Abnormalities in leaf morphology can result from disorder in either of the two or during both stages.

To determine which stage in leaf development process is primarily impaired in *atcsp3* loss of function mutants, I monitored palisade cell shape during early leaf development. The shape and size of the cells in early leaf primordia of 5th leaves from wild type (Col-0), *atcsp3-2*, and *atcsp3-1* plants were similar (Figure 1-10A). Cell number and cell size were not altered until 14 DAG when the leaf primordia expanded into adult leaves after major cell proliferation in 5th leaf blades (Figure 1-6B and C). All of the mutant alleles and wild type plants exhibited a cell size and cell shape that was nearly identical. However, the distance between individual cells in *atcsp3-2* was relatively less than wild type, resulting in a stacked and condensed cell layer appearance. Differences in cell size were observed at 21 DAG, where the cells complete the transition from the cell proliferation stage to the cell expansion stage. These data suggested that morphological differences in cell shape and size in *atcsp3-2* and *atcsp3-1* do not manifest until the end of cell proliferation. Thus, *AtCSP3* appears to primarily control leaf morphology at the leaf cell expansion stage through leaf cell polarity elongation. Similar to the epidermal cell shape as shown in Figure 1-9, palisade cells of 21 and 28 DAG leaves in *atcsp3-1* plants contain irregularly sized palisade cells. However, these irregularly sized cells were not observed in early expanded leaves (Figure 1-6F and I). This observation indicated that the uneven cell formation in *atcsp3-1* is initiated during the cell expansion stage and likely results in the generation of a curly leaf.

Expression of leaf polar expansion genes in *atcsp3* mutants

Morphological and anatomical analysis of *atcsp3* loss of function mutants suggested that AtCSP3 functions mainly in leaf-length polarity cell expansion to determine leaf shape. Therefore, I was interested to determine if the expression of leaf cell polarity specific genes is affected by the *atcsp3* loss of function mutation. To address this question, I monitored the transcript levels of several leaf polarity specific regulator genes at 28 DAG. Specifically, I determined mRNA abundance of *ROT3*, *LNG1*, and *LNG2* which are leaf-length directional regulators; *AN* and *AN3*, which are leaf-width directional regulators; and *CYCD3;1* which is a cell division regulator. I harvested total RNA from 5th leaves at 28 DAG for semi-quantitative RT-PCR analysis. *CYCD3;1*, which is a marker of cell division in leaf tissue, was not effected in *atcsp3-2* and *atcsp3-1* (Figure 1-7). Interestingly, changes were observed in transcript levels of leaf polar growth regulator genes (Figure 1-7). Among the leaf-length regulator genes, *LNG1* mRNA was reduced in *atcsp3-2* and *atcsp3-1* at 28 DAG. Other cell polarity specific regulators to leaf-length direction, *ROT3* and *LNG2*, were not changed in their mRNA transcript abundance compared to the wild type at the same time point. *LNG2* is a functionally redundant gene of *LNG1*, however, *LNG2* gene expression in *atcsp3-2* and *atcsp3-1* did not show the same pattern of gene expression as *LNG1*. These data are in accordance with the observation that loss of function of either *LNG1* or *LNG2* does not affect their transcription of each other (Lee 2006). However, *ROT3*, *AN3*, and *AN* did not show any alteration in their expression levels.

Taken together, these data support the hypothesis that *AtCSP3* affects transcript accumulation of genes involved in the regulation of leaf cell polarity expansion such as *LNG1*.

As described in the morphological and anatomic analyses, *atcsp3-1* plants had small orbicular leaves accompanied by a curly leaf surface. Thus, I studied the expression of genes involved in leaf flatness in the *atcsp3* loss of function mutant background. In the *atcsp3-1* mutant, leaves such as 3rd and 4th leaves became curled at approximately 14 DAG, which is 5 days later from the time of emergence of their leaf primordia from the shoot apex (Figure 1-10A). Other leaves, except 1st and 2nd leaves, also became curled approximately 5 days after the emergence of their leaf primordia. Therefore, I hypothesized that the genes responsible for the induction of curly leaf phenotype might be detectable with gene expression analysis approximately 5 days after the emergence of their respective leaf primordia. For this reason, I collected shoot apical tissue and leaf primordia of 7th, 6th and 5th leaves from plants at 14 DAG to

analyze the expression of genes involved in curly leaf phenotypes such as *TCPs* and *HASTY* (Qin 2005). Semi-quantitative RT-PCR showed that the transcript levels of *TCPs* and *HASTY* were not altered in the early stage of leaf generation (Figure 1-10B). I also checked the mRNA of these genes at 21 DAG and 28 DAG, but no changes in mRNA abundance were observed (data not shown). Although I could not qualify a relation between *AtCSP3* and the selected genes related to the control of leaf flatness, these data suggest that the curly leaf phenotype in *atcsp3-1* is affected by a mechanism which functions independently from TCP and HASTY gene regulation.

Subcellular localization of *AtCSP3* in onion epidermal cells

To determine the subcellular localization of the *AtCSP3* full length protein, I fused its complete coding sequence to the N-terminus of sGFP(S65T) which was driven by a CaMV 35S promoter (Figure 1-8 A). The sGFP(S65T) and *AtCSP3*:sGFP plasmids were bombarded into onion epidermal cells with a particle bombardment delivery system and cells transiently expressing GFP were visualized with confocal microscopy. Cells transformed with sGFP(S65T) alone showed both nuclear and cytosolic fluorescence (Figure 1-8 B). *AtCSP3*:sGFP transformed cells also fluoresced in both the cytosol and nucleus indicating that *AtCSP3* is a nucleocytoplasmic protein.

Discussion

***AtCSP3* expression is enriched in reproductive tissues**

AtCSP3 (At2g17870) is one of four Arabidopsis cold shock domain proteins (AtCSPs) containing an N-terminal cold shock domain (CSD) and C-terminal retroviral-like CCHC zinc fingers interspersed with glycine-rich regions. The four AtCSPs are highly similar at the amino acid sequence level (Karlson 2003) and have been recently characterized in relation to floral and silique development (Nakaminami 2009). *AtGRP2/CSDP2/AtCSP2* was recently characterized on a biochemical level (Sasaki 2007) and RNAi mutational analysis linked its function to plant development (Fusaro 2007).

To begin to understand the function of *AtCSP3* in plant development, we studied its expression patterns in different tissues with qRT-PCR analysis. Similar to *AtGRP2/CSDP2/AtCSP2* expression patterns, *AtCSP3* also accumulates more in tissues like shoot apices, inflorescence meristems, open flowers, and siliques compared to mature rosette leaves and stem tissue (Figure 1-1, Sasaki 2007, Fusaro 2007). These data are in good accordance to those obtained from the *AtCSP3* transcript abundance analysis results performed using microarray data by Genevestigator ([http:// www.genevestigator.ethz.ch/](http://www.genevestigator.ethz.ch/)) (data not shown) and those previously described (Nakaminami 2009).

***AtCSP3* loss of function generates stunted and orbicular leaf morphology**

T-DNA independent insertion alleles were selected for functional characterization and confirmed by semi-quantitative PCR analysis. Both *atcsp3-2* and *atcsp3-1* mutant alleles have 5' truncations in *AtCSP3* RNAs in the 5'UTR and the cold shock domain, respectively (Figure 1-2). The loss of function mutations in the *AtCSP3* gene resulted in smaller sized leaves, flower and siliques and an orbicular leaf shape in both mutant alleles (Figure 1-3). The most prominent characteristic for the loss of function mutants is the abnormal orbicular leaf shape that is accompanied by short petioles (Figure 1-4). Since *AtGRP2/CSDP2/AtCSP2* and *AtCSP3* exhibit high homology in their cold shock domain regions, I were interested to confirm whether *atcsp3* mutants also exhibit developmental related defects as described for *AtGRP2/CSDP2/AtCSP2* down-regulated plants. As previously mentioned, *AtGRP2/CSDP2/AtCSP2* RNAi mutants showed defects in flowering time, flower organogenesis, and seed development (Fusaro, 2007).

It is important to note that the authors did not mention abnormalities in vegetative tissue growth for the *AtGRP2/CSDP2/AtCSP2* knock-down mutants (Fusaro 2007). With the exception of shorter length flowers, *atcsp3* loss of function mutants did not exhibit significant abnormalities in flower organogenesis or flowering time. Taken together, our observations suggest that AtCSPs likely have diversified roles in Arabidopsis since mutant *atcsp3* phenotypes did not overlap with those described for the *AtGRP2/CSDP2/AtCSP2* gene.

Stunted and orbicular leaf shape in *atcsp3* loss of function mutants is promoted by leaf-length specific expansion and cell distribution in lateral leaf expansion

atcsp3 loss of function mutants exhibited an atypical orbicular leaf morphology. Recently, abnormal leaf shape has been investigated in different Arabidopsis mutant alleles by employing genetic and histochemical methods (for review Tsukaya 2007). In general, lateral leaf blade morphology is determined by the harmonious control of two-dimensional proliferation and expansion of leaf cells. Investigations of loss of function *rot3*, *rot4*, *an*, and *an3* mutant plants described a functional relationship between cell proliferation and cell expansion. As previously described, the small and rounded leaf morphology of *rot3* and *rot4* is caused in the leaf-length direction by alteration of cell proliferation and cell expansion, respectively (Narita 2004, Tsuge 1996). The rounded leaf shape of the *atcsp3* loss of function mutants is very similar to the *rot3* and *rot4* mutant phenotype (Narita 2004, Tsuge 1996). Even though *an* and *an3* gene mutants exhibited different leaf shapes than *rot3* and *rot4* mutants, they were also implicated in polarity specific regulation of leaf shape (Horiguchi 2005, Tsuge 1996). Therefore, the molecular mechanisms controlling cell proliferation and cell elongation during leaf blade morphogenesis appears to be complex. Image analysis of the wild type and *atcsp3* loss of function mutants (Figure 1-5) indicated that total cell number in *atcsp3* leaf blades is not affected, but the cell distribution is altered in two-dimensions. Our data demonstrated that cell numbers in the leaf-length axis are dramatically decreased, whereas cell numbers in the leaf-width axis are increased and cell sizes are smaller relative to the wild type (Figure 1-5H, I, and J). I hypothesize that these alterations result in the orbicular leaf phenotype in *atcsp3* mutants (Figure 1-4).

Leaf cell proliferation is primarily determined during early leaf generation stage and partially during two-dimensional lateral leaf growth. As showed in Figure 1-6, early lateral leaves of wild type and *atcsp3* loss of function mutants have similar palisade cell size, cell

numbers and cell shape. In addition, I did not observe any altered phenotype for dorsoventrality and determination of the leaf primordium cells. In contrast, as shown in Figure 1-6, the cell expansion during the late stages of early leaf generation is different in wild type and *atcsp3* loss of function mutants. These microscopic observations indicate that *AtCSP3* does not function in leaf primordia and during the early leaf expansion stage but it mainly affects two-dimensional cell expansion during lateral leaf expansion. Although the orbicular leaf phenotype in *atcsp3* loss of function mutants is caused by an alteration in two-dimensional cell expansion, the curly leaf phenotype of *atcsp3-1* cannot be explained only with defects in two-dimensional polarity cell expansion. In rosette leaves of *atcsp3-1*, epidermal cells have an entangled appearance in curly areas of leaf blades (Figure 1-5C and Figure 1-9C). In addition, palisade cells in mature leaves have an uneven cell size which differs from the wild type and *atcsp3-2* (Figure 1-5F). During early lateral leaf genesis, palisade cell expansion in *atcsp3-1* showed an irregular cell size and cell shape (Figure 1-6I). Therefore, it is possible that the physical force caused by the convergence of uneven sized and shaped cells at curled leaf areas breaks the normally flattened plane of the leaf blade surface. Taken together, microscopic analysis indicated that the *atcsp3-1* leaf is distorted by uneven leaf cell distribution which is accompanied by uneven leaf cell expansion.

***atcsp3* loss of function mutation alters *LNG1* gene expression**

Leaf shape and size determination is affected by cell differentiation on adaxial and abaxial leaf surfaces during early stages of leaf morphogenesis and two-dimensional leaf cell expansion (Barkoulas 2007, Kim 2006). Complicated temporal and spatial networks of many genes play important roles in determining general leaf morphology. An incomplete transition of leaf development from the shoot apical meristem results in abnormal leaf morphology with deformation in symmetry, polarity, and flatness (Heisler 2005, Benkova 2003, Ori 2000, Byrne 2000, Tiantis 1999, Timmermans 1999, Waites 1998, Long 1996). The *atcsp3* loss of function mutant did not exhibit phenotypic abnormalities during the leaf initiation stage. Our semi-quantitative RT-PCR data for *SHOOT MERISTEMLESS (STM)*, *ASYMMETRIC LEAVES1 (AS1)*, *CUC1* and *2 (CUP-SHAPED COTYLEDON 1 and 2)*, and *PIN-FORMED 1 (PIN1)* genes using shoot apex and early leaf tissues did not identify any alteration in the patterns of mRNA abundance (data not shown). These observations support the hypothesis that *AtCSP3* primarily

functions during lateral leaf development. Our histological observations, which determined that the abnormal leaf cell shape and size of *atcsp3* are initiated during lateral leaf expansion, are in good accordance with this scenario (Figure 1-6).

I demonstrated that the *atcsp3* loss of function mutation affects *LNG1* mRNA expression via an unknown mechanism. *LNG1* is a regulator of leaf-length polarity. (Figure 1-7). *LNG1* is an independent leaf cell expansion regulator of leaf length polarity and its expression is not affected by *ROT3* and *AN* (Lee 2006). Our RT-PCR data for *ROT3* and *AN* genes in *atcsp3* mutants is similar to those previously published in a study characterizing a *lng1* loss of function mutant. These data suggest that AtCSP3 does not function in relation to *ROT3* and *AN* during two-dimensional leaf expansion (Lee 2006). Overexpression of *LNG2*, which is a homolog of *LNG1*, results in a narrow and long leaf phenotype, was not altered in the *atcsp3* mutants. Lee *et al.* confirmed that a *lng1-3 lng2-1* double mutant has an additive effect in leaf-length cell expansion (Lee 2006). In addition, *LNG1* and *LNG2* expression was not affected in *lng2* and *lng1* mutants, respectively (Lee 2006). Thus, I hypothesize that AtCSP3 affects *LNG1* gene function only, resulting in reduced cell length along leaf-length direction and this regulation occurs independently of *LNG2* gene function.

Although the whole leaf phenotype of *atcsp3-1* looks more severe than *atcsp3-2*, the *atcsp3-2* mutant exhibited severe deformation in cellular shape and size resulting in an orbicular leaf shape phenotype. Although I cannot confirm why the two mutants have varying degrees in severity of leaf morphology mutations, it may result from differences in gene expression between the two alleles due to a positional effect of the T-DNA insertion. A future comparative microarray study between the two mutant alleles would enable us to gain a more precise understanding for this disparity.

AtCSP3 may play a role in stabilizing mature RNA of leaf shape determining genes

The reduction in expression of *LNG1* at 28 DAG in *atcsp3* mutants suggests that AtCSP3 positively affects their transcripts during leaf blade expansion. By what mechanism could AtCSP3 have an effect on *LNG1*? The CSD is a well conserved domain which facilitates binding to single and double stranded nucleic acids (Graumann 1998). Bacterial CSPs have an effect on both the transcriptional and translational level (Hofweber 2005) and in eukaryotes, YB-1 has pleiotropic functions in transcription and translation (for review; Ghono 2003). Typically,

RNA binding activity of YB-1 activates the translation of silent RNA for genes relating to cell proliferation, malignant transformation, and stress response (Evodokimova 2006). *Chlamydomonas* RNA binding protein NAB1 also regulates LHCBM mRNA stabilization, resulting in the induction of LHCBM protein translation (Mussegnug 2005). To date, all plant CSPs tested including OsCSP1, OsCSP2, *AtGRP2/CSDP2/AtCSP2*, AtCSDP1, and WCSP1 exhibit nucleic acid binding activity (Chaikam 2008, Fusaro 2007, Kim 2007, Nakaminami 2006, Karlson 2003). Subcellular localization of AtCSP3 shows that AtCSP3 is a nucleo-cytoplasmic protein similar to other RNA binding proteins (Figure 1-8). Therefore, it is reasonable to consider that AtCSP3 may exert a similar role and positively affects *LNG1* transcripts during leaf cell proliferation and expansion in the lateral leaf genesis stage.

Figure 1-1. Predicted secondary structure and tissue specific gene expression of *AtCSP3*

A) Domain architecture of *AtCSP3* protein.

B) Quantitative real-time PCR analysis of *AtCSP3* mRNA expression in different tissues. *Actin2* was used as an internal control for normalization. All data points for individual *AtCSPs* were calibrated with the respective normalized value of cDNA from root tips. The data shown represent the average and standard deviation of three replicates (elon. means elongation part; mat. means maturation part of root; S.A.M. abbreviates shoot apical meristem).

Figure 1-2. T-DNA insertion alleles of *AtCSP3*

A) Genetic map of T-DNA insertion positions within the *AtCSP3* locus. The white box represents the *AtCSP3* exon and black boxes represent 5' and 3' UTRs. With respect to the open reading frame, a SALK and Ds-Lox lines had insertions at -42 and +170 base pairs, respectively.

B) Semi-quantitative RT-PCR analysis of *AtCSP3* expression with RNA harvested from leaves 28 DAG of wild type (Columbia-0) and *AtCSP3* T-DNA insertion alleles. PCR was performed for the described cycle numbers with primers flanking the insertion (F3 or F2 and R1) and additional non-flanking region primers (F1 and R1). Arabidopsis actin 1 (AAC1) was used as an internal control. This figure is a representative image from three replicate reactions.

Figure 1-3. Morphological analysis of *atcsp3* loss of function mutants

A) Vegetative growth of the wild type (Col-0) and *atcsp3* insertion lines at 28 DAG grown under long day conditions (16/8 hrs for light/dark) at 23 °C. Flower bolts were removed to facilitate clear visualization of rosette leaf morphology. B) Total number of leaves of the wild type and *atcsp3* insertion mutants. Rosette leaves were taken from the same plant shown in panel (A) and leaves were aligned starting from the first leaf from cotyledons. Additional small developing leaves were harvested from the area surrounding the shoot apex. C) Comparison of flower size. Opened flowers were collected from primary bolts at 35 DAG (bar=0.5cm). D) Vertical views of flowers. E) Comparison of fully matured siliques collected at 48 DAG (bar=1cm). F) Seed size

comparison. All seeds were collected from the wild type and *atcsp3* insertion lines shown in E). G) Total leaves were counted from 14 to 49 DAG. Total leaves were numbered starting from the first leaf next to cotyledons exclusive of cauline leaves (n=16). All data show the average of total leaf number and error bars indicate standard deviation. H) Plant size of wild type Col-0 and *atcsp3* loss of function mutants. 28 DAG plants were grown in soil under long day light conditions and compared to each other. I) Root elongation measurement of the wild type and *atcsp3* loss of function mutants (n=20). J) Silique length comparison. The longest siliques were selected to determine silique length at 48 DAG (n=20). K) Seed count number. Seeds were collected from the same silique that was used for silique length measurement (n=20). All plots in I, J, and K show an average measured value and error bars represent standard deviation.

Figure 1-4. *atcsp3* loss of function mutants have short petioles and small-sized orbicular leaves. A) Comparison of leaf shape of 5th leaves from the cotyledons. 5th leaves were collected from 28 DAG plant, grown in soil under long day conditions at 23 °C (bar, 1 cm). Graphs in B, C, and D were plotted with the average leaf blade length, petiole length and leaf index of 5th leaves from 28 DAG plants, respectively. B) 2-dimensional measurement of leaf blade length. Leaf length was measured in the longitudinal (leaf length and lateral (leaf-width) directions from leaf epidermal cell images of a leaf blade by ImageJ software (n=20). C) Measurement of petiole length (n=20). D) Comparison of leaf index. Each leaf index was determined by the ratio of length to width in a leaf blade (n=20). A value close to 1.0 is indicative of increased roundness in leaf shape.

Figure 1-5. Orbicular leaf shape of *atcsp3* loss of function mutants is caused by impairment of cell growth polarity to leaf-length direction. Anatomical analysis was performed with 5th leaves of 28 DAG plants. Images of individual mutant alleles were taken from the same position in middle part of an adaxial leaf blade. Left end to right end of image depicts leaf-length direction and top to bottom depicts leaf-width direction. A-C) Propidium staining images of epidermal cell of wild type Col-0, *atcsp3-2*, and *atcsp3-1*, from left to right panels. D-F) Nomarsky images of palisade meshophyll cells of wild type Col-0, *atcsp3-2*, and *atcsp3-1*, from left to right panel (bar, 100 μm). Note that loss of function *atcsp3* mutants have small cell sizes relative to the wild type. G) Number of palisade cell on leaf blades. Total cell number was determined by counting the number of cells per unit area which is multiplied by the ratio between the original leaf area and observed leaf area (n=3). H) Count of palisade cell numbers

in two dimensional directions. Palisade cell number was counted along middle axes in two dimensional directions from DIC images (n=3). I) Length of individual epidermal cells in relation to length and width of leaves. Measurement of “ α ” shape epidermal cells was used to determine cell length in the leaf-length orientation. For measurements in relation to leaf-width, “ β ” shape cell were used (n=30 from three different leaves). J) Cell length of individual palisade cells in both directions. (n=30 from three different leaves). All data in graphs are average values with error bar indicating standard deviation.

Figure 1-6. Comparison of palisade cell expansion during lateral leaf generation of the 5th leaf.

DIC images were taken from the middle part of 5th leaves at different time points from wild type Col-0, *atcsp3-2* and *atcsp3-1*. Wild type Col-0, *atcsp3-2*, and *atcsp3-1* images are placed from left to right in a row. Upper row A), B), and C) are 14 DAG 5th leaf palisade cells from the adaxial surface of leaf blades. Middle row D), E), and F) are 21 DAG 5th leaves and bottom row G), H), and I) show 28 DAG 5th leaves. Scale bars indicate 100 μm.

Figure 1-7. Semi-quantitative RT-PCR analysis of genes related to leaf shape development.

Total RNA was extracted from 5th leaves of 28 DAG plants. With the exception of *AAC1* amplification, all PCR reactions were performed for 30 cycles with the described primer sets in Table 1-3. *AAC1* was used as an internal control and was amplified for 26 cycles. This figure contains representative results that were obtained among three replicates for each individual gene.

Figure 1-8. AtCSP3 localizes in the nucleus and cytosol in onion epidermal cell.

A) Schematic representation of sGFP(S65T) and *AtCSP3:sGFP* vectors. The coding region of *AtCSP3* was amplified by high fidelity PCR and sub-cloned into the sGFP(S65T) vector for transient expression via particle bombardment system. B) Transient analysis of subcellular localization for AtCSP3 in onion epidermal cells (bar, 100 μ M). Note the nucleocytoplasmic localization of AtCSP3.

Figure 1-9. Morphology of curly leaf shape in *atcsp3-1*. A) Comparison of leaf shape at 28 DAG (bar, 1cm). B) Side view of a 4th leaf at 28 DAG (bar, 1cm). C) Dark field microscopic images of epidermal cells in curly and flat adaxial leaf surfaces of 5th leaves in *atcsp3-1* at 28 DAG. Wild type Col-0 image displays the mid-portion of the 28 DAG leaf surface. This image indicates that the curled leaf surface contains entangled and irregularly shaped epidermal cells within the curly leaf area. Flat areas in *atcsp3-1* leaves contain cells with even size and shape.

Figure 1-10. Semi-quantitative RT-PCR analysis of leaf flatness-related genes in the early stage of lateral leaf generation at 14 DAG.

A) Whole plant morphology at 14 DAG. At this time, the 7th leaf is in a primordial stage and the 6th leaf is approximately 1 mm in length. 5th leaf length reaches to ~ 4 mm in the wild type and *atcsp3* mutant alleles (bar=1cm). B) Summary of semi-quantitative RT-PCR analysis for leaf flatness related genes. S denotes shoot apical meristem. 5, 6, and 7 are leaf numbers of 14 DAG plant. All PCR reactions, except *AAC1*, were performed with 30 cycles. *AAC1* was used as an internal control and was amplified with 26 cycles.

Table 1-1. Analysis of flowering time and seedling size

Genotype	Flowering time (DAG)	Height (cm)	Fresh weight (mg)	Sample size (n)
Col-0	22.5 ± 1	38.88 ± 1.28	395.5 ± 14.5	14
<i>atcsp3-3</i>	22.5 ± 0.90	38.58 ± 1.62	371.5 ± 17.8	16
<i>atcsp3-2</i>	23.25 ± 0.97	31.83 ± 2.53	256.5 ± 26.6	14
<i>atcsp3-1</i>	22.58 ± 0.79	31.33 ± 2.31	189.6 ± 13.7	12

Table 1-2. List of context sequence and amplicon length for Taq-Man probes

Primer name	Sequence (5'-3')	Amplicon length (b.p.)
Actin 2	CTGGATCGGTGGTTCCATTCTTGCT	85
AtCSP3	ATGATATGTGTGCTTGCTTATTAC	91

Table 1-3 Primer list for semi-quantitative RT-PCR

Primer name	Sequence (5'-- 3')
F1	TGGTTGTTTTGGTAGTTTTTG
F2	CGGTTTCATTACTCCCGATG
F3	GGGTTTTCATTTTACCCATTTC
R1	TCATTGCAAGGAAAGACAAGA
AAC1-for	CGGCGATTCCAGGGAACATTGG
AAC1-rev	GTGCTCGACTCTGGAGATGGTG
ROT3-for	AGATTTTCGTCAGCGGAAAGA
ROT3-rev	CCAAAGGGTGTGAAGCAAAT
AN-for	AAACCTGGGGCTTTTCTTGT
AN-rev	CCTGTTGCCTACTGGTGGAT
LNG1-for	ATG GAG AAG ACG CAGCATT
LNG1-rev	GACTGCTTCTCGAACCCAAG
LNG2-for	GAAGGAAGAGGAGCGGCTAT
LNG2-rev	CCGCTTCTGAATTTACCAT
AN3-for	GCTGGTTACTACCCAGCAA
AN3-rev	ATTCCAAGCTGGCTATGGTG
CYCD3;1-for	ATGGATGCTTCAGCTCGTTT
CYCD3;1-rev	GATGCGGTCCACTGGTAGTT

CHAPTER 2

Ectopic expression of Arabidopsis *AtCSP4* affects late stages of embryogenesis via alteration of endosperm development related gene expression

Abstract

Eukaryotic cold shock domain proteins are nucleic acid binding proteins that are involved in transcription, translation via RNA chaperone activity, RNA editing, and DNA repair during tissue developmental processes and stress responses. Eukaryotic cold shock domain proteins have been functionally implicated in important developmental transitions, including embryogenesis in both animals and plants. *Arabidopsis thaliana* Cold Shock Domain Protein 4 (AtCSP4; At2g21060) contains a well conserved cold shock domain (CSD) and glycine-rich motifs interspersed by two retroviral-like CCHC zinc fingers. GUS staining analysis in pAtCSP4:GUS transgenic *Arabidopsis* plants confirmed that *AtCSP4* was expressed in all tissues but accumulated in reproductive tissues and those undergoing cell divisions. Overexpression of *AtCSP4* reduced silique length and embryo lethality. Interestingly, a T-DNA insertion *atcsp4* mutant did not exhibit any phenotypes, implicating that the similar *AtCSP2* gene is functionally redundant with *AtCSP4*. During silique development, overexpression of *AtCSP4* induced early browning and shrunken seed formation beginning with the late heart embryo stage. A fifty percent segregation ratio of the defective seed phenotype was consistent with the phenotype of endosperm development gene mutants. Transcripts of *FUS3* and *LEC1* genes, which regulate early embryo formation, were not altered in the *AtCSP4* overexpression lines. On the other hand, transcripts of *MEA* and *FIS2* which are involved in endosperm development were affected by overexpression of *AtCSP4* indicating that *AtCSP4* may be a regulator of endosperm development via transcriptional or post-transcriptional regulation. Additionally, overexpression of *AtCSP4* also affected the transcript abundance of several MADS box genes in stages of early silique development. Specifically, the transcripts of *AP*, *CAL*, *AG*, and *SHP2* were up-regulated. Collectively, these results indicate that *AtCSP4* plays an important role during the late stages of silique development by affecting the expression of several development related genes.

Introduction

Cold shock domain proteins (CSPs) are among the most conserved nucleic acid binding proteins known, whose members are identified in bacteria, plants, and animals. CSPs participate in various cellular functions, which are mediated by their characteristic ability to bind nucleic acids (for review: Braun 2000, Evdokimova 2001, Kohno 2003, Wilkinson 2001). CSPs are mainly implicated to function pleiotropically during organism development and they are well-

studied in relation to cold stress responses in bacteria. In humans, the Y-box binding protein 1 (YB-1), is transferred from cytosol into the nucleus in response to specific physiological, environmental and growth stimuli. YB-1 functions in transcription regulation, DNA repair, and mRNA splicing (Faustino 2003, Kohno 2003, Raffertseder 2003), thereby effecting organism development. Under normal conditions, YB-1 is localized in the cytoplasm and functions as RNA chaperone in cytoplasmic RNPs and mediates translational repression (Bader 2005, Evdokimova 2001). Mouse YB-1 is expressed during embryogenesis and a YB-1 deficient mutant exhibits an embryo lethal phenotype (Lu 2005). MSY4, another mouse CSP, is also highly expressed during embryogenesis and can rescue a YB-1 loss of function mutant in early embryogenesis (Lu 2006). Similar to animal CSPs, cold shock domain proteins from plants such as Arabidopsis and rice have also been implicated in development (Nakaminami 2009, Chaikam 2008, Sakai 2007, Fusaro 2007). Among the four Arabidopsis CSPs, AtCSP2 (AtGRP2/CSDP2) is characterized for its function in affecting flowering time and reproductive tissue development including seed development (Fusaro 2007). Further studies are necessary to understand the functions of AtCSPs in relation to plant reproductive development.

Embryogenesis primarily establishes the future body plan of the shoot-root system in angiosperms. Zygotic embryogenesis consists of two main phases: 1) embryo morphogenesis or formation, which determines plant organ development and 2) maturation phase or postembryonic development, which induces organ expansion and storage of metabolites for seed germination. Embryo morphogenesis fulfills apical-basal formatting, shoot and root meristem generation and cell differentiation during early embryogenesis (Laux 2004). In the maturation phase or postembryonic development, the mature embryo is surrounded by lipid and storage substances which are important for seed germination and desiccation tolerance (Raz 2001). Physiology and genetics of the embryogenesis process has been well studied (for reviews: Park 2008, Willemsen 2004, Laux 2004, Jurgens 2001, Goldberg 1994). After fertilization, the fertilized zygote divides to establish a position-dependent root and shoot meristem, which determines the fate of the plant body post-germination (Mayer 1998). The mature seed consists of the embryo and endosperm, whose pattern formation and future developmental plan are decided by a specific gene regulatory network (for review: Laux 2004). The transcriptional regulation of embryo formation is initiated specifically from the first asymmetric division of the fertilized single precursor cell, resulting in a primary apical-basal embryo. This apical and basal embryo development is genetically

regulated by *WUSCHEL-RELATED HOMEODOMAIN (WOX) 2* and *WOX 8* genes, respectively (Haecker 2004). *GNOM/EMB30*, *PINFORMED7 (PIN7)*, *PIN1*, *GURKE*, *FAKLE (FK)*, *monopteros (mp)* genes also regulate apical-basal embryo formation (Friml 2003; Gälweiler 1998; Geldner 2003, 2001; Mayer 1993, 1991; Schrick 2000; Berleth 1993).

Post embryonic development is controlled by the *LEAFY COTYLEDON (LEC)* class of genes which include *LEC1*, *LEC2*, *FUS3*, and *FUSCA3* genes (Parcy 1997; Lotan 1998; Luerßen 1998; Stone 2001). Loss of function mutants for each of these genes exhibit defects in the storage of nutrients that are required for germination and desiccation tolerance during seed dormancy. During late embryogenesis in Arabidopsis, endosperm development is a pivotal process for seed maturation. Four genes belonging to the Fertilization Independent Seed (FIS) family repress endosperm maturation in non-pollinated and non-fertilized embryos (for review: Köhler 2006). The FIS family Polycomb group (PcG) proteins include *MEDEA (MEA/FIS1)*; Grossniklaus 1998, Kiyosue 1999), *Fertilization Independent Seed 2 (FIS2)*; Luo 1999), *Fertilization Independent Endosperm 3 (FIE/FIS3)*; Ohad 1999), and *MULTI-COPY SUPPRESSOR OF IRA1 (MSI1)*; Köhler 2003a, Guitton 2004). Loss of function mutants of FIS genes result in two distinct phenotypes depending on pollination and fertilization. In the absence of pollination, loss of function mutants can initiate seed development. In contrast, in the presence of pollination and fertilization, embryogenesis is arrested at the heart stage of embryo development, resulting in embryo lethality (For review: Chaundhury 2001; Chaundhury 1997, Peacock 1995, Grossniklaus 1998, Ohad 1996). Proteins of FIS family assemble to form an FIS complex, which functions in the repression of endosperm development genes by parental and maternal imprinting in Arabidopsis (for review: Köhler 2006, Gehring 2004). A MEA loss of function mutant results in 50 % embryo lethality in a heterozygous mutant line which is not rescued by a multi-copy as well as a single copy of the wild type MEA allele, indicating that MEA functions only from the female gametophyte for embryogenesis (Grossniklaus 1998, Chaundhury 1997). *PHERES1 (PHE1)*, which encodes a MADS box protein that is imprinted in maternal chromatin, is repressed by the FIS complex, including MEA (Vielle-Calzada 1999, Köhler 2003a, Köhler 2003b). FIE and MEA proteins form a multimeric complex, which modifies chromatin structure thereby inducing the transcription of target genes (Spillance 2000). Using a transgenic DNA methyltransferase-1 gene antisense plant, DNA methylation of

chromatin was shown to affect parent-of-origin effect and plays a primary role in endosperm development (Luo 2000).

MADS box proteins have been extensively studied in relation to their functional role in floral organ identity determination and transition of the floral meristem. The ABC model was developed to explain the overlapping functions of several MADS box proteins during floral organ formation (for review: Robles 2005, Jack 2004). *APETALA1* (*AP1*) and *APETALA2* (*AP2*) belongs to the A class which are important for the generation of sepals and petals. *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) belong to the B class of genes forming petals and stamens. The C class consists of the *AGAMOUS* (*AG*) gene, which is involved in stamen and carpel development. In addition, the *SEPALLATA* (*SEP*) gene was represented in both B and C class floral organ development (Honma 2001, Pelaz 2000, Coen 1991). In parallel to seed maturation via embryogenesis, siliques also develop as an organ for storing developing seeds (for review: Roeder 2006). MADS box genes included in the ABC model are expressed highly during silique development and embryogenesis (Lehti-shiu 2005, Folter 2004, Alvarez Buylla 2000, Parěnicová 2003). Gene expression of *AG*, which is a carpel development gene, especially increases again during fruit development and ripening (for review: Seymour 2008). Also, *SHATTERPROOF* (*SHP*) and *FRUITFULL* (*FUL*) are expressed highly and are functionally important during silique development. (Folter 2004, Liljegren 2000, Ferrandiz 2000, Gu 1998). Redundant functions were identified for *AP1* and *FUL* genes during floral meristem identity determination, however their function diverges during fruit development (Ferrandiz 2000). *CAULIFLOWER* (*CAL*) also functions in floral meristem identity determination along with *AP1*. Many *AGAMOUS*-like proteins are also highly expressed during fruit development (Lehti-Shiu 2005). Especially, *AGAMOUS like-15* (*AGL15*) and *AGAMOUS like-18* (*AGL18*) are expressed in both endosperm and embryo tissue (Alvarez Buylla 2000, Heck 1995). A double loss of function mutant, *agl15/agl18* flowers early but not single mutants, implicating that *AGL15* and *AGL18* act redundantly to regulate floral transition (Adamczyk 2007). Constitutive overexpression of *AGL15* in *Arabidopsis* delays leaf and flower senescence as well as fruit maturation (Fernandez 2000). *AGL15* was recently shown to bind *AtCSP2* and *AtCSP4* promoters using a Chromatin immunoprecipitation (ChIP) assays. These data support the hypothesis that *AtCSP* genes are regulated by MADS box proteins, and that *AtCSPs* may have a functional role during silique development (Nakaminami, 2009).

In the present study, we performed morphological analyses in T-DNA insertion and gain of function *AtCSP4* mutants. Overexpression of *AtCSP4* impairs normal silique size determination and embryo development. In addition, the expression of several MADS box genes and endosperm development genes are altered in *AtCSP4* overexpression lines. These data suggest that *AtCSP4* affects the silique development and embryogenesis by affecting genes involved in silique and seed development.

Methods and Materials

Plant materials and growth conditions

A GABI-Kat T-DNA insertion mutant of *AtCSP4* was obtained from GABI-Kat (Stock number: GK 623B08.01, www.gabi-kat.de) and Col-0 wild type seeds were purchased from Lehle Seeds (Round Rock, TX). Seeds were stratified at 4 °C for 4 days under dark conditions. All plants were grown in Metromix 360 soil (Scott Co., Marysville, OH, USA) under long day conditions at 23 °C (16 hr/8hr for light/dark cycle). To generate *AtCSP4* overexpressed lines, we amplified the coding region of *AtCSP4* with gene specific primers from Col-0 wild type plant with KOD-Hot High Fidelity Taq Polymerase ; forward primer 5'-CAC CAT GAG CGG AGG AGG AGA CGT GAA C-3', reverse primer 5'-ACG AGC ACC ACC GCT AGT GCA ATC CCT TGC-3' (Novagen, Gibbstown, NJ, USA). The amplified coding sequence of *AtCSP4* was cloned into the pENTR entry vector plasmid according to standard procedures (Rubio 2005, GATEWAY; Invitron Carlsbad, CA). pN-TAP binary vector (NTAPa) was obtained from ABRC (<http://www.arabidopsis.org>, stock number:CD3 696). The *AtCSP4* gene sequence was then transferred from pENTR into the NTAPa vector using LR reaction (Invitrogen, Carlsbad, CA). The resultant plasmid was designed as *35S:NTAP:AtCSP4*. To confirm sequence integrity and reading frame, the construct was analyzed by DNA sequencing (Macrogen, Rockville, MD). This construct was transformed into the GV3103 *Agrobacterium* strain using a MicroPulser electroporator with an electronic pulse of 2.4 kV, 25 uF for 5 milliseconds according to the manufacturers instructions (Biorad, Hercules, CA). The *35S:NTAP:AtCSP4* gene construct was transformed into *Arabidopsis* by the floral dip method (Clough 1998). Transgenic plants were selected on 1× Murashige and Skoog (MS) plates including Gamborg's Vitamins, 1 % phytoagar, 1 % sucrose and 25 µg/ml gentamycine (Caisson Labs, North Logan, UT).

Gene expression analysis

For characterizing gene expression in the *atcsp4* T-DNA insertion mutant and overexpression lines of *35S:NTAP:AtCSP4*, total RNA was extracted from leaf tissue using TRIzol® reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using 500 ng of total RNA with the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA). Semi-quantitative RT-PCR analysis was performed with the Go-Taq Flexi PCR reaction kit (Promega, Madison, WI). Thermocycling conditions for determining *AtCSP4* transcript levels were as follows : 95 °C for a 2 min initial denaturation followed by 95 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec, for a specified number of cycles (as described within individual figures) followed by a final extension step for 7 min at 72 °C. Primers are listed in Table 1-2.

For *AtCSP4* transcript measurement in different tissues, we utilized Taq-Man probe qRT-PCR analysis with TaqMan® Universal PCR Master mix (Applied Biosystems, Foster City, CA). Primers and TaqMan® probes for *AtCSP4* and *ACT2* genes were purchased from Applied Biosystems and primer sequences are described in Table 1-3; with the names NM_180280.1 (*ACT2*) and NM_127676.2 (*AtCSP4*). Thermocycling conditions used are as follows: 95 °C for 10 min followed by 50 cycles of 95 °C for 15 sec and 60 °C for 1 min. Total RNA from different tissues, except leaves, were extracted with the Plant RNA extraction Reagent (Invitrogen, Carlsbad, CA). cDNA was prepared as described above.

Total RNA of siliques was extracted from different stages of silique development to study the transcript levels of MADS box and embryogenesis related genes using the Plant RNA extraction Reagent (Invitrogen, Carlsbad, CA). Total RNA was reverse transcribed to cDNA using the same protocol as described above. All thermocycling was performed as follows with the described cycle number written within figures: 95 °C for 2 min, each cycle comprising 95 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 30 sec, with a final extension step for 7 min at 72 °C. Primers for each gene amplification are described in Table 1-4.

GUS expression plant and histological analysis

To generate a GUS expression gene construct fused to the *AtCSP4* promoter, a 2 kb fragment upstream of the start codon of the *AtCSP4* gene was amplified by KOD Hot Start DNA polymerase (Applied Biosystems, Foster City, CA). Primers were designed using genomic

sequence information containing KpnI and NcoI restriction sites. The following primers were used: forward 5'-TCT GGT ACC GGG AAA AAC CCA CGC TTG -3' and reverse 5'-TCT CCA TGG TCA CCG TTC CCT TGC GTC -3'. The amplified DNA fragment was digested by restriction enzymes and subsequently ligated into the pCAMBIA1303 binary vector in-frame with the GUS gene. This vector was transformed into the LBA4404 *Agrobacterium* strain by a MicroPulser electroporator as previously described. Transformation of this vector into Col-0 wild type *Arabidopsis* was performed by the floral dip method (Clough 1998). Transgenic *Arabidopsis* was screened on 1× MS containing 1 % sucrose, 1 % phytoagar containing 25 µg/ml of hygromycin.

GUS staining was performed on non-fixed tissues harvested from various developmental stages. Collected tissues were incubated quickly in GUS staining buffer (50 mM Na₂HPO₄, 30mM NaH₂PO₄, 0.4mM K₃[Fe(CN₆)], 0.4mM K₄[Fe(CN₆)], 8mM EDTA, 0.1 % Triton X-100, 10 % methanol, and 1 µg/ml 5-bromo-4-chloro-3-indol-glucuronide cyclohexylamine salt (X-Gluc)), overnight at 37 °C. Stained tissue was washed several times in 70 % ethanol and samples were subsequently observed under a Nikon SMZ-U dissecting microscope equipped with a Nikon DXM 1200 CCD camera (Melville, NY, Nikon).

Seed clearing and microscopy

Seeds were dissected from different silique developmental stages and cleared in modified Hoyer's solution (80 % chloral hydrate, 20 % glycerol, 10 % water by volume) for 2 hours. Cleared seeds were mounted on slide glass and images were acquired with a Nikon ECLIPSE E600 differential interference contrast (DIC) microscope equipped with Nomarski optics and Nikon DXM 1200 CCD camera system (Nikon, Melville, NY).

Subcellular localization of AtCSP4

The coding region of the *AtCSP4* gene was amplified with the following primers: 5'-TCTGTCGACATGAGCGGAGGAGACGT-3', and 5'-AGACCATGGTACGAGCACC-ACCGCTAGTGC-3' containing the *NcoI* and *Sall* restriction enzyme digestion sites, respectively. Amplified PCR products were digested with these restriction enzymes and ligated into a pre-digested sGFP(s65T) plasmid. Sequence integrity and maintenance of the reading frame were confirmed by DNA sequencing. Five µg of sGFP empty plasmid or sGFP-*AtCSP4*

plasmids were coated onto DM-10 Tungsten particles which were prepared as according to the manufacturer's instructions (BIO-RAD, Hercules, CA). A Biolistic® PDS-1000 particle bombardment system was used for transient transformation of onion cells by employing 25 inches of Hg vacuum, a 1000 psi rupture disk, and a 12 cm target distance (Hercules, CA, BIO-RAD). Bombarded onion epidermal cell layers were incubated at 22 °C for overnight under dark conditions. DIC and GFP images were obtained by a Zeiss Axioimager LSM-501 confocal microscope and analyzed with LSM image analysis software (Carl Zeiss AG, Germany).

Results

Characterization of the *AtCSP4* gene

AtCSP4 (*AtGRP2b*; At2g21060) and *AtCSP2* (*AtGRP2*; At4g38680) are highly homologous to one another and encode smaller sized proteins than the other two *AtCSPs* (*AtCSP3*; At2g17870 and *AtCSP1/CSDP1*; At4g36020). The *AtCSP4* gene encodes a 603 base pair transcript which does not contain introns. The *AtCSP4* is a small sized protein with a highly conserved N-terminal CSD and three repeated glycine-rich domains which are interspersed by two C-terminal CCHC zinc finger motifs (Figure 2-1 A). *AtCSP4* shows 86 % of amino acid homology to *AtGRP2* and their CSD regions are highly conserved (Figure 2-1 B). Due to this high similarity in sequence, it is possible that *AtCSP4* may function redundantly with *AtCSP2*.

To determine if *AtCSP2* expression is affected by *AtCSP4*, we studied *AtCSP2* transcript levels in a T-DNA insertion mutant and *AtCSP4* overexpression plants (Figure 2-3A and C). As expected, the T-DNA insertion mutant and overexpression plants showed a loss and accumulation of *AtCSP4* transcript, respectively (Figure 2-1 C). In order to assess if *AtCSP4* levels affect *AtCSP2* transcript accumulation, we also tested for alterations of *AtCSP2* transcript abundance and none were detected. These data indicate that *AtCSP4* and *AtCSP2* do not participate in any feedback regulation for one another.

AtGRP4 expression analyses

The Genevestigator (<http://www.genevestigator.ethz.ch/>) public microarray database confirmed that the *AtCSP4* mRNA is present in all tissues of Arabidopsis but is enriched in reproductive and meristematic regions. *AtCSP4* and *AtCSP2* transcripts are expressed at higher

levels relative to the two additional *AtCSPs* (Nakaminami 2009). Comparative analysis of *AtCSP4* expression using microarray and real-time PCR data confirmed that *AtCSP4* transcript is highly abundant in reproductive tissues, especially in carpels and siliques. To characterize the tissue specificity of the *AtCSP4* transcript, we employed a Taq-Man probe quantitative real-time PCR (qRT-PCR) assay. As shown in Figure 2-2A, the *AtCSP4* mRNA is expressed in all tissues similar to the pattern of *AtCSP2/AtGRP2* (Nakaminami 2009, Fusaro 2007). The *AtCSP4* gene transcript is much less abundant in mature leaf tissues where cell divisions are reduced and tissue identity has already been determined. In contrast, the *AtCSP4* transcript preferentially accumulates in reproductive and meristematic tissues such as inflorescences and shoot apices. *AtCSP4* is highly abundant in mature siliques harboring full grown seeds.

To monitor the tissue specific gene expression of *AtCSP4* *in planta*, we developed transgenic plants by transforming wild-type plants with the T-DNA binary vector containing the *AtCSP4* promoter fused to the GUS reporter gene (Figure 2-2B). We used T2 generation transgenic plants for GUS expression assays. GUS staining results revealed that *AtCSP4* is expressed in all tissues in good accordance to data obtained by qRT-PCR analysis (Figure 2-2A). Specifically, the *AtCSP4* promoter is highly active in reproductive and meristematic tissues. In root tissues, root tip areas of primary and lateral roots were strongly stained, while root vascular tissues were weakly stained (Figure 2-2, B and C). At 21 DAG, lateral root tips contained high levels of GUS (Figure 2-2D). In young seedlings at 7 DAG, the hypocotyl contained strong GUS gene expression relative to other tissues (Figure 2-2C). Tissues exhibiting high cell divisions such as young leaves, leaf primordia, and shoot apices showed high GUS gene expression (Figure 2-2D). In leaf blades, GUS expression was especially accumulated at the trichome base on the younger leaf surface area. In contrast, mature and older leaves no longer maintained high levels of GUS expression. Vascular tissue also exhibits GUS expression in young leaves but its expression disappears gradually following leaf expansion. Root vascular tissue also exhibits high levels of GUS gene expression at early stages of root development (Figure 2-2C). The shoot apical meristem exhibits high activity of the *AtCSP4* promoter, resulting in dark blue staining in the center of shoot apices from both primary and lateral shoots (Figure 2-2F) and the inflorescence meristem (Figure 2-2G). Reproductive tissues exhibit stronger *AtCSP4* promoter activity relative to vegetative tissues (Figure 2-2F and H) with floral tissues exhibiting strong GUS expression during the early stages of floral organ development.

Floral organs such as carpels, sepals, and stamens exhibited high GUS gene expression, whereas sepals did not (Figure 2-2H). Siliques with mature seeds showed the highest level of GUS gene expression relative to any other tissue (Figure 2-2I). Specifically, the dehiscence area, two valves, and matured, but not ripened seeds, in siliques stained very strongly (Figure 2-2I).

Taken together, the qRT-PCR and GUS staining results indicate that *AtCSP4* is expressed in multiple tissues but accumulates preferentially in meristematic and reproductive tissues. Lastly, *AtCSP4* is highly expressed in silique tissue comprising matured seeds.

Morphological analysis of *AtCSP4* overexpression and T-DNA insertion mutant

To characterize *AtCSP4* gene function *in planta*, we functionally characterized an *AtCSP4* overexpression and T-DNA insertional mutant line. The T-DNA insertion line was obtained from the GABI collection (GK-623B08.01). A homozygous line was obtained and the precise location of the T-DNA insertion was determined with sequence analysis with a T-DNA flanking and gene specific primer pair. From these genotyping results, we confirmed that the T-DNA insertion occurs within the cold shock domain coding region at +91 base from the first initiation codon (Figure 2-3A). When total RNA was isolated and converted into cDNA, *AtCSP4* transcript was not detected in the GK-623B08.01 with a primer pair that was designed to amplify full length *AtCSP4* (Figure 2-3B: F1+R1). To confirm if this line is a null mutant allele of *AtCSP4*, we performed semi-quantitative RT-PCR with non-flanking region primers, whose complementary positions are denoted in Figure 2-3A. Interestingly, an *AtCSP4* gene fragment was amplified with primer pairs that did not flank the T-DNA insertion and which targeted the 3' UTR (Figure 2-3B). However, when a forward primer was used which flanked the T-DNA insertion in combination with the 3' UTR specific primer, no product was amplified (Figure 2-3B). Therefore, these data confirm that the GK-623B08.01 T-DNA insertion line is not a null allele of *AtCSP4*, but instead it generates truncated *AtCSP4* mRNA.

We generated an overexpression line of *AtCSP4* fused to an N-terminal TAP-tag that was driven by a 35S promoter (*35S:NTAP:AtCSP4*) as a means to observe the functional effect of ectopic overexpression of *AtCSP4* (Figure 2-3C). We obtained 17 different gentamycin resistant lines in the T1 generation. Four representative lines showing embryo lethal and abortive phenotypes are presented in Figure 2-4. *AtCSP4* transcript accumulation in these lines was

determined by semi quantitative RT-PCR, which confirmed elevated levels of *AtCSP4* transcript in overexpression lines relative to the wild type.

To gain insight into the function of *AtCSP4*, we compared the phenotypes of T-DNA insertion mutant and overexpression lines of *AtCSP4* to the wild type under long day light conditions. As shown in Figure 2-3E, the *atcsp4* T-DNA insertion mutant and the *35S:NTAP:AtCSP4* overexpression lines exhibits typical phenotypes during shoot and leaf tissue development. The seedling height of both homozygous *atcsp4* and heterozygous *35S:NTAP:AtCSP4* lines did not differ from those observed in the wild type under long day conditions (Figure 2-3E and F). In addition, total leaf number and leaf size did not differ from wild type (Figure 2-3G). With respect to flowering time, all lines initiated flowering at 23 DAG similar to wild type plants (data not shown). Therefore, overexpression or T-DNA insertional mutation of *AtCSP4* does not appear to affect vegetative development and flowering time. Even though, we did not identify any atypical phenotypes during vegetative growth, we observed brown and shrunken seeds within mature siliques of *35S:NTAP:AtCSP4* overexpression lines. We were unable to obtain *35S:NTAP:AtCSP4* homozygous plants, even after screening up to the T4 generation using gentamycin resistance as a selectable marker (data not shown). These data indicated that the embryo lethal phenotype may arrest normal seed development in *35S:NTAP:AtCSP4* homozygous lines. Therefore, we compared reproductive tissue morphology of heterozygous *35S:NTAP:AtCSP4* lines from the T3 generation to those of the homozygous T-DNA insertion mutant *atcsp4* and the wild type. We used the *35S:NTAP:AtCSP4-3* line as a representative line of *35S:NTAP:AtCSP4* for all the tests. The interval between individual silique position on the primary stem of *atcsp4* and *35S:NTAP:AtCSP4* was identical to the wild type (Figure 2-4A). Floral shape and size of *atcsp4* and *35S:NTAP:AtCSP4* were not altered (Figure 2-4B). However, the length of mature siliques in the *35S:NTAP:AtCSP4* line was shorter than that of the wild type and *atcsp4* (Figure 2-4C and D). In addition, the embryo lethal phenotype was always associated with overexpression lines which developed short siliques.

To examine the embryo lethality seeds *35S:NTAP:AtCSP4* plants, we observed the external seed shape and seed formation in mature siliques. As shown in Figure 2-4C, all four of the *35S:NTAP:AtCSP4* lines showed short siliques, harboring embryos that were either aborted or embryo lethal seeds. The extent of embryo lethality varied among the the four lines. Specifically, the *35S:NTAP:AtCSP4-12* line possessed white immature seeds as well as embryo

lethal seeds. *35S:NTAP:AtCSP4-3* and *4-8* lines showed very similar distribution of embryo lethal seeds and the *35S:NTAP:AtCSP4-1* line contained has the lowest number of embryo lethal seeds. The proportion of the *35S:NTAP:AtCSP4-3* plants generating short silique harboring embryo defective seed to the plants generating wild type silique and seeds is 54.9 % : 45.1 % in the T3 generation plant which was grown in soil without antibiotics selection (n=36). We also counted the number of defective seeds and normal wild-type seeds in short siliques to determine the segregation ratio in self fertilized T3 generation plants of *35S:NTAP:AtCSP4-3*. The total number of defective seed and wild-type seed in the plants showing an embryo lethal phenotype segregated to 51.66 % : 49.34 % (Table 2-1). However, the *atcsp4* T-DNA insertion mutant contained typical silique morphology and seed maturation. Taken together, the *AtCSP4* gene does not appear to be an essential gene for completion of the reproductive stage. This hypothesis is further supported by the fact that *AtCSP4* is 1000-fold down-regulated in *Ler* ecotype relative to *Col-0* (Nakaminami 2009). Ectopic overexpression of *AtCSP4* induces short siliques that harbor about 50 % embryo defective seeds. Therefore, overexpression of *AtCSP4* impairs normal seed and silique maturation via an unknown functional mechanism.

Image analysis of zygotic embryo development

We conducted further studies to identify the precise embryogenesis stage which stage is impaired and which part of the seed is damaged by overexpression of *AtCSP4*. Seeds were cleared and extracted from early maturing stages of siliques derived from *35S:NTAP:AtCSP4-3*, T-DNA insertion mutant *atcsp4*, and the wild type . We referred to each silique stage according to Dr. Bowman's definition for determination of the stages of embryo development (Bowman 1994, www.genomeforschung.uni-bielefeld.de/GF-research/AtGenExpress-Seedssiliques.html). As shown in Figure 2-3, the *atcsp4* T-DNA insertion mutant exhibits normal embryo and endosperm development in seeds. No morphological defects were observed in the external seed shape and size in *atcsp4* mutants. Since *35S:NTAP:AtCSP4-3* contains has two different seed phenotypes, embryo lethal and wild type seeds within the same silique, we carefully observed all seeds until the embryo defective phenotype appeared. Seeds in *35S:NTAP:AtCSP4-3* plants developed normally until the heart stage of embryogenesis (Figure 2-5 K and L). No abnormalities were observed in *35S:NTAP:AtCSP4-3* until the early heart stage. In comparison to the wild type, endosperm expansion occurred normally in the overexpression plants until this stage. However,

after the heart stage, *35S:NTAP:AtCSP4-3* seeds no longer advance to the torpedo stage and endosperm started to shrink at the same stage and the seed coat rapidly turned brown (Figure 2-5M and N). In contrast, seeds from the *atcsp4* T-DNA insertion mutant and the wild type sustained green color and normal shape (Figure 2-5N and O). The other seeds in *35S:NTAP:AtCSP4-3* plants continued growing normally and did not differ morphologically from the wild type. Thus, we conclude that the embryo lethal seeds in *35S:NTAP:AtCSP4* result from defects in the late heart stage embryo and immature endosperm development during seed maturation.

Embryogenesis related genes and MADS box protein gene expression during early silique development stages

Seed development is initiated after fertilization by the mating pollen and ovule. Seeds are developed from a gynoecium in the carpel and the carpel grows into a silique which harbors developing and mature seeds. To elucidate the relationship between defective seed maturation and related gene expression in the *35S:NTAP:AtCSP4-3* line, we studied the gene expression levels of both initial floral organogenesis genes and seed development related genes in *35S:NTAP:AtCSP4* plants. As shown in Figure 2-5, zygotic embryogenesis of *35S:NTAP:AtCSP4-3* is impaired from the late heart stage. The late heart stage embryo comprises the silique stage 4, according to Dr. Bowman's definition of silique development (Bowman 1994, www.genomeforschung.uni-bielefeld.de/GF-research/AtGenExpress-Seedssiliques.html). Public microarray data of silique development revealed that *AtCSP4* peaks in its gene expression at the carpel developmental stage, which occurs during early silique development (<http://www.genevestigator.ethz.ch/>). Therefore, we collected floral buds and early developmental stages of siliques to monitor gene expression of candidate genes that function in silique development and seed embryogenesis. Among the representative regulators of embryo formation during early embryogenesis, *FUS3*, *LEC2*, and *ABI3* were amplified at same number of PCR cycles using cDNA from floral buds, and silique stage 1, and stage 3 tissues from wild type and *35S:NTAP:AtCSP4-3* plants. The transcripts of *MEA*, *FIS2*, *FIE*, and *PHE1* genes were studied in these tissues with the cDNA prepared from RNA extracts from *35S:NTAP:AtCSP4* plants. As shown in Figure 2-6 A, *FUS3* and *LEC2* genes were not altered in their transcript in overexpression plants in the tissues we studied relative to the wild type. We could not detect the

ABI3 transcripts in our amplification assays even after 40 cycles of amplification (data not shown). Among the genes involved in endosperm development, *MEA* mRNA was highly accumulated during the early stage of embryogenesis in *35S:NTAP:AtCSP4-3* plants (Figure 2-6A). Previous studies indicated that in wild-type plants, *MEA* transcript was detected in inflorescences and open flowers before fertilization and it gradually disappears after the initiation of embryogenesis. Our semi-quantitative RT-PCR results showed the expected gene expression that is consistent with previously reported data in the wild type (Figure 2-6A, Baroux 2006). In contrast, the *MEA* transcripts in *35S:NTAP:AtCSP4-3* was dramatically reduced relative to that of the wild type during seed development (Figure 2-6A). Two other endosperm developmental genes, *FIS2* and *FIE* were accumulated their mRNA at similar levels in floral buds in the wild type and *35S:NTAP:AtCSP4-3*, but the *FIS2* mRNA was less expressed during stage 3 in *35S:NTAP:AtCSP4-3*. Collectively, results of semi-quantitative RT-PCR analysis of embryogenesis related-genes suggest that the mRNA accumulation of endosperm development genes such as *MEA* and *FIS2* was affected during floral bud and early stages of silique development in *35S:NTAP:AtCSP4-3* plants, with *MEA* mRNA accumulation being the most affected during embryogenesis. Transcripts of *LEC1*, *FUS3*, *ABI3*, and *FIE*, an endosperm development gene, were not altered during early embryogenesis stages in *35S:NTAP:AtCSP4-3*, indicating that those genes are not likely to contribute to the defective seed phenotype observed in *35S:NTAP:AtCSP4-3* plants.

To understand the relationship between the impairment of silique development (shortened size) and development of abnormal seeds, I studied the expression of genes that are involved in silique development in *35S:NTAP:AtCSP4* plants. MADS box proteins have been functionally implicated in early silique development, ovule development, and seed embryogenesis (Lehti-Shiu, 2005). Since one of the characteristic mutant phenotypes of *35S:NTAP:AtCSP4* plants was a shortened silique length, we monitored the expression of MADS box protein genes in developing floral and silique tissue. I also determined the transcript expression of pistil and fruit development genes such as *FUL* and *SHP2*. In floral buds, the transcripts of *API*, *CAL*, and *AG* genes accumulated more in *35S:NTAP:AtCSP4-3* plants relative to the wild type (Figure 2-6B). During the silique stage 1 and mature flower stage, *SHP2* mRNA was also transcribed at higher levels than wild type. Similar observations were made for mRNA generation of *API*, *CAL*, and *AG*. In silique stage 3 samples, most of the evaluated genes, with the exception of *SHP2*,

recovered to same level of expression as in wild type. The *SHP2* transcripts in *35S:NTAP:AtCSP4-3* accumulated more at silique stage 3. However, *FUS* transcript abundance was not altered in all of the tested stages relative to the wild type. Therefore, overexpression of *AtCSP4* affects the mRNA accumulation of *API*, *CAL*, and *AG* during the floral organogenesis phase starting from floral buds. However, those aforementioned genes are not altered in expression during silique development. I propose that short siliques generated *35S:NTAP:AtCSP4-3* are induced by increasing transcripts of several MADS box protein genes at the onset of the early phases of silique development.

Subcellular localization of AtCSP4 in onion cell

The sub-cellular localization of AtCSP4 was examined by making a C-terminal fusion of sGFP(S65T) to AtCSP4, which is driven by CaMV 35S promoter (Figure 2-8A). The 35S:sGFP and 35S:AtCSP3:sGFP plasmids were transformed into onion epidermal cells by particle bombardment. 35S:sGFP was expressed in both nucleus and cytosol (Figure 2-8B) and 35S:AtCSP4:sGFP transformed onion cells also exhibited localization specific to cytosol and nuclei.

Discussion

Putative redundant function of AtCSP4/AtGRP2b and AtCSP2/AtGRP2

Plant CSP genes have been functionally characterized in wheat, tobacco, rice, and Arabidopsis but their function remains elusive (Sakai 2007, Chaikam 2008, Fusaro 2007, Karlson 2003, Nakaminami 2006 and 2009). Arabidopsis possesses four CSPs which can be sorted into two groups based on the protein length and amino acid identity; AtCSP2 and AtCSP4 proteins contain ~200 amino acids and AtCSP3 and AtCSP1/CSDP1 contain ~300 amino acids. The amino acid identity and similarity between AtCSP2 and AtCSP4 is 86% and 95%, respectively (Figure 2-1B). Detailed tissue specific expression analyses confirmed that both of these genes are highly expressed in meristemic and reproductive tissues (Nakaminami 2009). Although the *AtCSP4* transcript is less abundant than *AtCSP2*, *AtCSP4* exhibited a similar trend of expression during development (Nakaminami 2009). Comparative analysis of gene expression between the *Ler* and Col-0 ecotype revealed that *AtCSP4* expression is 1000-fold reduced in *Ler*

relative to the Col-0 ecotype, suggesting that a loss of AtCSP4 is not critical for plant survival. This hypothesis is further supported by T-DNA mutant analysis where knock-out of the AtCSP4 gene does not appear to have adverse effects on plant growth and development (see Figure 2-3). Interestingly, when *AtCSP4* is overexpressed, severe development affects are manifested. *AtCSP2* transcript is unaffected in *atcsp4* knockout and overexpression mutant lines. Thus, the functional interactive relationship between *AtCSP4* and *AtCSP2* is not clearly understood at this time.

Our qRT-PCR data for *AtCSP4* is in good accordance with a previous report (Figure 2-2A, Nakaminami 2009). GUS expression driven by the *AtCSP4* promoter also confirmed that *AtCSP4* is predominantly expressed in meristemic and reproductive tissues, especially in siliques (Figure 2-2B). These GUS data are also in good accordance with *AtCSP2* gene expression data previously obtained *in situ* hybridization and GUS detection assays (Nakaminami 2009, Fusaro 2007, Sasaki 2007). However, no atypical phenotypes were observed in an *atcsp4* T-DNA insertion mutant. These data contrast those previously observed for an RNAi mutant lines of *AtCSP2* (Figure 2-2, Fusaro 2007). Taken together with the RNAi results and the 1000-fold reduction of *AtCSP4* in *Ler* ecotype, the lack of any aberrant phenotype in vegetative and reproductive tissues in the *atcsp4* T-DNA insertion mutant strongly suggests that *AtCSP2* may have greater functional importance *in planta* (Figure 2-3, 4 and 5; Nakaminami 2009, Fusaro 2007). Furthermore, similarities in expression patterns of *AtCSP4* and *AtCSP2* suggests that *AtCSP2* may function redundantly with *AtCSP4* and complements the *atcsp4* mutant. Most functionally characterized plant CSPs have been suggested to function as RNA binding proteins based on the results of nucleic acid binding assays in wheat and Arabidopsis (Sakai 2007, Fusaro 2007, Kim 2006). In the case of winter wheat WCSP1 and Arabidopsis CSDP1, RNA chaperone activity has been proposed on the basis of *in vivo* and *in vitro* assays (Nakaminami 2006, Kim 2006).

Ectopic overexpression of *AtCSP4* reduces silique lengths and induces embryo lethality

A homozygous *atcsp4* T-DNA insertion line generates truncated mRNA of *AtCSP4*. In addition to characterizing this knock-out line, I also developed four 35S promoter driven Arabidopsis for overexpression of *AtCSP4* which all exhibit same phenotype. The *atcsp4* T-DNA insertion mutant does not exhibit any atypical phenotype (Figure 2-3). Flowering time and

whole seedling size did not differ from those observed in wild type plants. Unlike the *atcsp4* T-DNA insertion mutant, the RNAi mutant of *AtCSP2* showed early flowering and abnormal flower generation (Fusaro 2007). On the other hand, overexpression of *AtCSP4* resulted in atypical phenotypes in reproductive tissues such as shortened silique size and defective seed maturation although developmental timing of vegetative and reproductive tissues was not affected by ectopic overexpression of *AtCSP4* (Figure 2-3). Positions of silique formation on the stem were also not altered (Figure 2-4A and B). In heterozygous *35S:NTAP:AtCSP4-3* plants, siliques contained ~50 % defective seeds (Table 2-1). These mutants resemble the phenotype observed for a FIS gene family mutant with impaired endosperm development (Grossniklaus 1998). For this parent-of-origin effect, the embryo lethal seed phenotype in a heterozygous *mea* mutant, a representative FIS gene, also shows a 50 % proportion in self-fertilization. The other *fis* mutants such as *mis1* also exhibited similar ratio of defective seed (Köhler 2003). Embryo formation in *35S:NTAP:AtCSP4-3* is also arrested at the late heart stage which is the same time point where defects in embryo formation in observed for a *fis* mutant allele (Figure 2-5; Ingouff 2005, Kiyosue 1999, Grossniklaus 1998, Chaudhury 1997, Ohad 1996). Therefore, it is reasonable to hypothesize that the defective seed in overexpression of *AtCSP4* results from a parent-of-origin effect on the endosperm development process.

Expression of MADS box and seed embryogenesis related genes are altered by overexpression of *AtCSP4*

Microscopic observation of embryos in seeds of *35S:NTAP:AtCSP4-3* revealed that early shrinkage of endosperm is established from the late heart stage (Figure 2-5). These clearing data indicate that overexpression of *AtCSP4* affects embryogenesis. CSPs from animal systems have also been implicated in embryo development. For example, mouse *YB-1* and *MSY4* proteins are expressed highly during embryogenesis and a double knock-out in mice cells prevent cell senescence, suggesting both genes share function in proliferative tissue in higher vertebrates (Lu 2006). In Arabidopsis, ChIP analysis confirmed that *AGL15*, a well-studied MADS domain protein expressed in developing embryos, binds to the promoter regions of *AtCSP4* and *AtCSP2*. These correlative data suggest that *AtCSPs* may also play an important role in silique development similar to their animal counterparts (Nakaminami, 2009). Therefore I are interested to further understand the functional relationship of plant CSP's during silique development. As

shown in Figure 2-5, the early embryo formation and endosperm development in *35S:NTAP:AtCSP4-3* is not retarded before late heart embryo formation stage. However, its embryo growth ceased from the late heart stage of embryo formation, which is almost the final stage of embryo cell divisions, and accompanied with a retardation of endosperm expansion (Figure 2-5, Laux 2004). In *35S:NTAP:AtCSP4-3*, expression of early embryo formation genes such as *FUS3*, and *ABI3* were not altered, while the expression of *MEA* and *FIS2* that are involved in endosperm development were affected during early seed development stages. Collectively, the defective seed in *35S:NTAP:AtCSP4-3* may be caused by an impairment of late embryogenesis and endosperm development. MEA, FIE, and MSI1 make a huge complex (~650 kDa) which may combine with FIS2 (Köhler 2003, Chanvivattana 2004). A loss of function mutant of the FIS gene perturbs endosperm development in a similar manner that is consistent with overexpression of *AtCSP4*. Gene expression of MEA and FIS2, which are FIS class PcG in Arabidopsis, is increased after fertilization in the *AtCSP4* overexpression line (Figure 2-6). MEA functions in relation to DNA methylation in the chromatin complex for sustaining paternal gene imprinting. MEA silencing in vegetative tissues is mediated by H3K27 methylation (Jullien 2006a, Jullien 2006b). Therefore, the cascade of gene regulation from *AtCSP4* to *MEA* may affect the gene methylation to develop endosperm.

MADS box proteins commonly function in relation to floral transition, fruit development, and ovule development (Seymour 2008, Lehti-Shiu 2005). Silique development is established from carpel identity determination which is regulated by *AG*. *API* and *FUL* function redundantly in floral meristem identity determination but their function is divergent in relation to lateral development involving fruit development (Ferrandiz 2000). The *ful* mutant generates very a small silique size because of defects in silique valve cell differentiation (Ferréndiz 2000). Also, *FUL* interferes with *SHP1* and *SHP2* gene expression, but *AG* induces their gene expressions in early silique development stage. *API*, *CAL*, *AG*, and *SHP2* except *FUL* gene were affected by ectopic overexpression of *AtCSP4*. However, all the MADS box genes that were tested in this study did not exhibit altered expression patterns at silique stage 3, when globular embryo formation is initiated. Therefore, I propose that regulation of MADS box protein genes by overexpression of *AtCSP4* affects majorly silique development but not embryogenesis.

As mentioned before, the expression of the *MEA* gene, which encodes a PcG protein, is also decreased at the floral bud stage, with a dramatic decrease at silique stage 3. That is, the

normal silique generation is impaired from floral meristem identity determination for carpel organogenesis. However, it is not clear whether the silique morphology and seed embryogenesis affects each other causing the *35S:NTAP:AtCSP4-3*'s phenotype with these data. Judging from previously published phenotype of various MADS box protein mutants, I presume that overexpression of *API*, *AG*, and *SHP2* does not result in embryo lethality (Liljegren 2000 and 1999, Mandel 1995, Mizukami 1992). Taken together with morphological and semi quantitative RT-PCR analyses, shortened silique and embryo lethality may be resultant from an independent alteration of gene transcripts for MADS box protein and endosperm development related genes.

Figure 2-2. Tissue specific gene expression of *AtCSP4*

A) Quantitative real-time PCR analysis of *AtCSP4* mRNA expression in different tissues. All data points for individual *AtCSPs* were calibrated with the respective normalized value of cDNA from root tips. The presented data represent the average and standard deviation of three replicates. *Actin2* was used as an internal control for normalization.

B) pAtCSP4:GUS expression in transgenic Arabidopsis. A -2 kb promoter region of *AtCSP4* was inserted into the pCAMBIA1303 binary vector (pAtCSP4:GUS).

C) pAtCSP4:GUS expression in early seedling of transgenic Arabidopsis. 7 DAG seedlings, D) 21 DAG, E) lateral root from 21 DAG, F) Inflorescence meristem from 28 DAG, G) Floral bud and early flower from 35 DAG plant, H) Flower organ of 35 DAG plant, I) Fully matured silique of 42 DAG plant, rectangle indicates modified full mature seed and dehiscence of the same silique. GUS staining was performed from seedlings grown on MS plates (C-E) or soil (F-I) under long day conditions. Scale bar indicates 0.5 μ m.

Figure 2-3. Morphological phenotype of a T-DNA insertion allele and overexpression of *AtCSP4* in vegetative tissues

A) Genetic map of T-DNA insertion positions within the *AtCSP4* locus. A white box represents the *AtCSP4* exon and black boxes represent its 5' and 3' UTRs. With respect to the open reading frame, a GABI-Kat line has an insertion at +91 base pairs. B) Semi-quantitative RT-PCR analysis of *AtCSP4* expression in leaves from 28 DAG of wild type (Col-0) and *AtCSP4* in the GABI-Kat T-DNA insertion line. T-DNA flanking and gene-specific primers positions were denoted in panel A. PCR was performed for the described cycle numbers with primers flanking the insertion and additional non-flanking primers. *AAC1* was used as an internal control. All semi-quantitative RT-PCR figures are representative images from three replicate reactions. C) Gene construct of *35S:NTAP:AtCSP4*. D) Semi-quantitative RT-PCR analysis of *AtCSP4*

expression in leaves from 28 DAG of the wild type (Col-0) and three different independent *35S:NTAP:AtCSP4* transgenic lines exhibiting the defective seed phenotype. E) Comparison of whole plants at 42 DAG. All plants were grown in soil under long day conditions (bar=2cm). F) Total leaf number in the wild type, *atcsp4*, *35S:NTAP:AtCSP4-3* plants. Leaf number was counted weekly post germination (n=30). All plots show mean and error bars represent standard deviation .

Figure 2-4. Morphological phenotype of the T-DNA insertion allele and overexpression of *AtCSP4* in Arabidopsis vegetative tissues

All plants used in this test were grown in soil under long day conditions. A) Silique formation on the primary stem at 49 DAG (bar, 2cm). B) Comparison of flower size. Opened flowers were collected from primary bolts at 35 DAG (bar, 0.2cm). C) Comparison of silique size. Fully matured siliques were detached from 49 DAG (bar, 1cm). D) Silique size comparison. Silique size were determined with the longest siliques from 50 different plants from the wild type and *atcsp4*, and 35S:NTAP:AtCSP4-3, respectively (n=50). E) Seed number comparison. Seed were counted from the same siliques which were used in panel D. All plots in D and E show mean $\pm$

standard deviation. F) Seed formation and maturation of the wild type and *atcsp4*, and T3 generation of *35S:NTAP:AtCSP4-3*, *1*, *8*, and *12* lines. All siliques were opened from fully matured silique at 49 DAG (bar, 0.5cm).

Figure 2-5. Comparative analysis of stages of silique development in the T-DNA insertion and overexpression lines of *AtCSP4*. Seeds were detached and cleared from 49 DAG siliques. (A-E) wild-type seeds, (F-J) *atcsp4*, and (K-O) *35S:NTAP:AtCSP4-3*. (A, F, and K) show globular stage embryos, (B, G, and L) show early heart stage embryos, (C, H, and M) show late heart stage embryos, (D, I, and N) are torpedo stage embryos and (E, J, and O) show the bent cotyledons (bar, 100μm).

Figure 2-6. Semi-quantitative RT-PCR analysis of genes involved in silique development and seed embryogenesis. A) Semi-quantitative RT-PCR analysis of MADS box genes. *AP1*, *CAL*, *AG*, and *SHP2* were affected by overexpression of *AtCSP4* in floral bud (FB) and stage 1. B) Semi-quantitative RT-PCR of seed embryogenesis related genes. *LEC1* and *FUS3* were used as marker genes related to embryo development. *MEA*, *FIS2*, and *FIE* were used as marker genes related to endosperm development. Overexpression of *AtCSP4* affect endosperm development genes such as *MEA* and *FIS2* but not *FIE*. FB means floral bud and arabic numbers denote silique stages. *AAC1* was used as an internal control.

Figure 2-7. AtCSP4 subcellular localization in onion epidermal cell. A) Schematic representation of *sGFP* and *AtCSP4:sGFP* vectors. The coding region of *AtCSP4* was cloned into the *sGFP*(S65T) vector for transient expression via a particle bombardment system into onion epidermal peels. B) Transient analysis of subcellular localization for *AtCSP4* in onion epidermal cells (bar, 125 μ m). *AtCSP4* localized to the cytosol and the nuclei.

Table 2-1. Segregation of homozygous *atcsp4* and heterozygous *35S:NTAP:AtCSP4-3* plant

Genotype of plant	Defective seeds	Wild type seed	Total seed number analyzed	Silique number analyzed
Col-0	0.58%	99.42%	868	20
<i>atcsp4</i>	0.69%	99.31%	874	20
<i>35S:NTAP:AtCSP4-3AtCSP4-3</i>	51.66 %	49.34%	2228	50

Table 2-2. Primer list for AtCSP4 transcript detection and amplicon length of PCR product with the R1 primer

Primer name	Sequence (5'-3')	Amplicon length (b.p.)
F1	CACCATGAGCGGAGGAGGAGAC	777
F2	AGAAGGGGTTTGGTTTCA	704
F3	TCACACCTAGCGACGGTG	686
F4	ATTTCGTAGCCTCGCAGC	625
F5	CGTCCCAAGGCTATTGAA	564
F6	TTCATCTGGTGGACGCGG	496
F7	AAGCTACGGAGGAGGTTA	433
F8	CATGGCGAGAGAATGCTC	340
F9	GGGTAAATCAGTCATCGATCC	150
R1	TTTCTCAAATCACAAGGAACCA	0

Table 2-3. List of context sequence and amplicon lengths for Taq-Man probes

Primer name	Sequence (5'-3')	Amplicon length (b.p.)
Actin 2	CTGGATCGGTGGTTCCATTCTTGCT	85
<i>AtCSP4</i>	TAATTAGGACTTGTGGTTGTTATGG	142

Table 2-4. Primer list for semi-quantitative RT-PCR analysis

Primer Name	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
AAC1	CGGCGATTCCAGGGAACATTGTGG	GTGCTCGACTCTGGAGATGGTGTG
AP1	CTTACGCCGAAAGACAGCTT	ACTGCTCCTGTTGAGCCCTA
CAL	TAGCAGGCTTAAGGCCAAGA	GTTGTGGCTGTGGTACATCG
AG	AGGCAATTGATGGGTGAGAC	CTAACTGGAGAGCGGTTTGG
FUL	GGGGGAAGATCTTGATTCGT	AGTTTGGTTCCGTCAACGAC
SHP2	CGACGCAATGGTTTACTCAA	CGGACACGACTGATTCCTTT
FUS3	GTGGCAAGTGTTGATCATGG	CGTGAAAACCGTCCAAATCT
LEC1	TTGAACTTGACCAGCACAG	AAGCTTGCTCATAGCCCAAA

MEA	TTGCTGCTAATCGTGAATGC	TCATCAACTTGGCGTAGCAG
FIS2	GCTTGATTCCATGTGGAGGT	CTGTGCCACGAGGTAAGTCA
FIE	TTGTGGGTCATGGAGATTCA	TGTGGGGAATTTTGATGGAT

CHAPTER 3

AtCSP1 regulates germination timing promoted by cold temperature.

Abstract

Cold shock domain proteins (CSPs) have been reported to play an important role in tissue development and cold responses. Eukaryotic CSPs play a crucial role in cell differentiation and cell proliferation which result in regulating the timing of tissue development and cell division. Cold shock domain proteins have been identified in many plants, although functional analyses have been limited to *Arabidopsis*, wheat and rice and their *in vivo* functional roles remain unclear. Among the four *Arabidopsis thaliana* CSPs (AtCSPs) that I have characterized, AtCSP1 is highly similar to AtCSP3 in terms of its predicted amino acid sequence. Transcription of *AtCSP1* increased in the loss of function mutant *atcsp3*, implicating that AtCSP3 negatively affects AtCSP1 transcription. Using a gene trap line (GT606 defined as *atcsp1*), which has complete loss of full length *AtCSP1* transcript, GUS gene expression was detected preferentially in tissue primordia and highly dividing tissues. The *atcsp1* exhibited early germination after stratification but did not exhibit any further atypical phenotype in vegetative tissues. Germination of *atcsp1* without stratification also occurred earlier than wild type but the germination time delayed 24 hours. Comparative analysis of GUS expression in seeds with or without stratification confirmed that *AtCSP1* expression was affected by cold temperature during radicle emergence. In addition, ABA germination assays revealed a reduced sensitivity to ABA in *atcsp1*. Taken together, AtCSP1 may function to regulate germination timing which is in turn mediated by cold temperatures to promote embryo expansion.

Introduction

The *Arabidopsis thaliana* model system contains four cold shock domain proteins (AtCSPs) that are being actively studied for characterization of *in planta* functional roles. As reported in other organisms, AtCSPs are involved in cold stress responses, growth and development (Sasaki 2008, Kim 2007, Fusaro 2007). By using nucleic acid binding assays, AtCSPs have been characterized as RNA binding proteins but their *in vivo* function roles have not been clearly elucidated. *AtCSP1*, which is a low temperature stress responsive gene, was previously shown to rescue the cold sensitive mutant phenotype of an *E. coli* mutant strain (BX04) which lacks four endogenous cold shock proteins (Kim 2007). Overexpression of *AtCSP1* in *Arabidopsis* was recently shown to delay germination timing under dehydration and

salt stress conditions (Park 2009). However, the function of AtCSP1 beyond the seedling developmental stage is not yet well understood.

The germination of seedlings is an important stage for studying early seedling development. Seed germination is controlled by the balance between the maintenance of seed dormancy and the establishment of seed germination. The turning point between seed dormancy and seed germination is determined by external environmental factors such as light, temperature, time after ripening and internal factors such as phytochromes, hormones and chemicals (Bentsink 2008, Holdsworth 2008). Seed dormancy is a special strategy that viable seeds employ to survive under unfavorable conditions (Finch-Savage 2006). Dry storage of ripened seeds and stratification by a moist chilling treatment are very important factors for breaking seed dormancy. Impairment in the formation of Arabidopsis seed coat such as endosperm and testa layer also affects embryo dormancy (for review: Bentsink 2008). In addition, the potential of the embryo to protrude into the micropylar endosperm layer is another seed structural factor which decides embryo dormancy (for reviews: Bentsink 2008, Kucera 2006). Due to the difference in timing between environmental stimuli and the breakage of embryo dormancy in dried seeds, it is very difficult to assess embryo dormancy. To overcome this limitation, dormancy assays are regarded as germination assays because the germination commences simultaneously with the breakage of seed dormancy (Bentsink 2008).

Seeds that are exposed to environmental factors such as cold temperatures, light, and long term storage at dry conditions, readily break seed dormancy. Also, several chemicals such as KNO_3 and NO_3 promote germination. These factors function interdependently to complete germination. For example, light interacts with low temperature in seed germination. However, none of these factors strongly promote germination (Cone 1983). Among these dormancy release factors, nitrate is the most sensitive followed by cold and light (Finch-Savage 2007).

Germination is defined as a process that establishes water uptake into dried seeds and results in radicle protrusion (Bewley 1994). Water uptake consists of three stages, rapid initial uptake, plateau phase, and a final further increase of water uptake during early germination (Schopfer 1984, Bewley 1997, Menz 2005). Following water uptake, a part of the seed layer is penetrated by a physical force which is defined as two stages of testa and endosperm rupture (Karssen 1976, Hopher 1985, Leubner-Metzger 2003, Liu 2005). The radicle which emerges from the intact embryo completes the germination process. The period of cold treatment and

darkness also affects the seed germination rate (Cutler 1996, Meng 2008, Kim 2008). Genetic mutants which exhibit impaired germination phenotypes during early germination stages are identified by germination tests which use the immature seeds that are detached from newly generated siliques (Raz 2001).

Light is an important environmental factor that induces seed germination and breaks dormancy. Phytochrome (*Phy*) mediated photocontrol is also a major factor that promotes germination by breaking seed dormancy. Five phytochromes have been identified in *Arabidopsis* and the loss of their light response affects seed germination (Donohue 2008, Castillon 2007, Sharrock 1989). Casal and Sánchez defined three different modes formed by inter-dependent interactions of five *Phy* genes in regulating the timing of germination (Casal 1989). These modes are generally defined as very low-fluence response (VLFR), low-fluence response (LFR), and high-irradiance response (HIR). Mutants lacking response to different spectrums exhibited alteration in the timing of germination. *PhyB* functions by red light sensitivity, therefore, mutants lacking *PhyB* do not exhibit normal seed germination (Shinomura 1994). Mutants lacking *PhyA* also lack seed germination without seed imbibition but germinated with imbibition (Shinomura 1994). . These observations indicate that red/far-red reversible LFR is mediated by *PhyB* while *PhyA* functions in VLFR in controlling seed germination (Shinomura 1996). However, double mutants of *phyA/phyB* did not exhibit any non-germination phenotype, implicating that other potential phytochromes also mediate seed germination (Poppe 1997, Yang 1995). Light also influences seed maturation that subsequently affects seed germination in specific genotypes (Munir 2001).

Cold temperature is required for the breakage of seed dormancy which in turn affects the phytochromes' role in seed germination (Donohue 2007). Pre-cold treatment with moisture, defined as stratification, is very important to establish seed germination because cold treatment establishes hormone synthesis such as gibberellin (GA) and abscisic acid (ABA) which induce seed germination (for review: Holdsworth 2008). GA is an essential phytohormone that enhances germination and can also replace the imbibition effect by cold and light (Derkx 1993). The endosperm monolayer, which potentially controls germination, is regulated by nitric oxide (NO) which subsequently affects GA signaling (Müller, 2006). The weakening of the endosperm is originally regulated by the embryo and it is regulated by an interaction between GA and ABA. GID, a GA receptor, DELLA domain proteins, and SCF^{SLY1} function in seed germination via

correlation with GA perception (Nakajima 2006, Sun 2004, McGinnis 2003). DELLA domain proteins consist of five members that include GAI, RGA, RGL1, RGL2 and RGL3 which repress GA responses during development (Sun 2004). Among them, RGL2 strongly repress seed germination but others showed a weak influence on germination (Cao 2005, Koornneef 1985). The correlation between DELLA proteins and environmental factors was proposed by Cao *et al.* (Cao 2005). Mutants lacking all four DELLA proteins did not show light and cold sensitivity required for seed germination. The GA biosynthesis gene (*GA3OX*) is also involved in seed germination. Among four genes of *GA3OX*, *GA3OX1* and *GA3OX2* play important roles in seed germination (Mitchum 2006). SPATULA (SPT) and PIF3-like 5 (PIL5), bHLH transcription factors, have been recently identified as light-stable repressors of seed germination and mediate temperature responses (Penfield 2005). Proteasome degradation of PIL5 is induced by red and far-red light stimuli followed by an induction of germination, implicating that PIL5 interacts with phyA and phyB (Oh 2006 and 2004). Red light suppresses ABA biosynthesis gene expression and induces ABA inactivation gene expression, such as *CYP707A2*, which promotes seed germination (Seo 2006). PIL5 activates ABA biosynthetic genes and represses an ABA degradation gene that results in the inhibition of seed germination (Oh 2007). Therefore, GA plays an antagonistic role with ABA to allow seed germination under environmental signal such as light and cold temperatures.

ABA is the classic repressor of seed germination (Finch-savage 2006). A general mechanism of ABA function in repressing seed germination is correlated with the induction and maintenance of seed dormancy (Carrera 2008). Since seed dormancy is established from seed maturation, embryogenesis is an interesting developmental process that decides the potential of seed germination. Mutant phenotypes of embryogenesis-related genes such as *ABA-INSENSITIVE3 (ABI3)*, *FUSCA3 (FUS3)*, and *LEAFY COTYLEDON (LEC1 and LEC2)* exhibit decreased dormancy during the seed maturation stage, meaning that loss of function of these genes induces early germination by impairment of seed dormancy (Raz 2001). Many mutants of them showed insensitivity to ABA function in ripened or moist chilled seeds (Nishimura 2007, Pandey 2006, Saez 2006, Yoine 2006, Katagiri 2005, Zhang 2005, Hugouvieux 2001, Xiong 2001). Functions of these loci have been related to RNA translation and metabolism, protein chaperone modification, protein kinase activation and microRNA regulation during seed germination. In protein chaperone modification, the E3 ligase ABI3-interacting protein 2 (AIP2)

and a component of the 26S proteasome (RPN10) act as regulators of seed germination (Smalle 2003, Kurup 2000). The ABI5-binding protein (AFP), which degrades ABI5 showed ABA hypersensitivity during seed germination (Lopez-Molina 2003). Recently, two protein kinases, SNF1-related protein kinase (SnRK2.2) and SnRK 2.3, have been identified and a double mutant showed strong ABA insensitivity for germination resulting in early germination (Fujii 2007).

Herein, I describe the early germination phenotype of a Gene trap line (*atcsp1*) inserted within the *AtCSP1* coding region. The correlation between *atcsp1* and moist chilling for seed germination is discussed. Also, I show that *AtCSP1* may function as a regulator in the timing of tissue differentiation by affecting the initiation of seed germination.

Methods and Materials

Plant materials and growth condition

The gene trap line was obtained through the Cold Spring Harbor Laboratory (Stock number: CSHL_GT606, <http://genetrapp.cshl.org>). Ler wild type seeds were purchased from Lehle Seeds for comparative analyses in a wild type background (Round Rock, TX). Seeds were stratified at 4 °C for 4 days under dark conditions and grown in Metromix 360 (Scott Co., Marysville, OH, USA) under long day conditions at 23 °C (16 hr/ 8hr for light/dark). For germination tests, GT606 seeds were surface sterilized with 0.01 % Triton/50 % bleach for 7 min followed by 7 rinses with distilled water. These seeds were sowed on 1 × Murashige and Skoog (MS) plates, including Gamborg's Vitamins, 1 % phytoagar and 1 % sucrose under continuous light conditions (Caisson Labs, North Logan, UT).

Determination of transcript abundance

For confirmation of the gene knock-out of *AtCSP1*, total RNA was extracted from leaf tissue of GT606 (*atcsp1*) and *Ler* using TRIzol® reagent (Invitrogen, Carlsbad, CA). We also extracted RNA from an *atcsp3* (Wisc_DsLox353G12) T-DNA insertion mutant for comparative analysis of gene expression. cDNA was synthesized using 500 ng of total RNA by the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA). Semi-quantitative RT-PCR was performed with five times diluted cDNA with the Go-Taq Flexi PCR reaction kit (Promega, Madison, WI). Thermo cycling conditions to understand the redundancy between *AtCSP3* and

AtCSP1 as follows : 95 °C for 2 min for an initial denaturation followed by 95 °C for 30 sec, 58 °C for 1min, 72 °C for 30 sec, for a total of 35 cycles (as denoted in individual figures) followed by a final extension for 7 min at 72 °C. Sequences of the primers used are as follows: for *AtCSP3* amplification, forward 5'-CCC ATT TCC TCT CTT TGT ACG-3', reverse 5'-TCA TTG CAA GGA AAG ACA AGA-3', for *AtCSP4* forward 5'-CAC CAT GGC TTC AGA GGA TCA ATC G-3', reverse 5'-TGA TGA GGT CAA AAT TTC CAG A-3'. Thermocycling conditions for Figure 2 were set as follows: 95 °C for 2 min for an initial denaturation followed by 95 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec, for specified cycles as denoted in individual figures, followed by final extension for 7 min at 72 °C. The primers used are as follows: F1- 5'-CAC CAT GGC TTC AGA GGA TCA ATC G-3', F2- 5'-AAT TGC CTG GTC CTT TTG GT-3', and R1 – 5'-TGA TGA GGT CAA AAT TTC CAG A-3'. *AAC1* was amplified similarly with the exception of a different cycle number. The primer sequences for *AAC1* amplification are a forward primer of 5'- CGG CGA TTC CAG GGA ACA TTG TGG -3' and a reverse primer of 5'- GTG CTC GAC TCT GGA GAT GGT GTG -3'.

GUS staining histological analysis

For GUS staining in young seedlings, tissues were collected from plants grown on MS plates as described above. Reproductive tissues were detached from the plants grown in soil under long day conditions. Collected tissues were incubated quickly in GUS staining buffer (50 mM Na₂HPO₄, 30mM NaH₂PO₄, 0.4mM K₃[Fe(CN)₆], 0.4mM K₄[Fe(CN)₆], 8mM EDTA, 0.1 % Triton X-100, 10 % methanol, and 1 µg/ml 5-bromo-4-chloro-3-indol-glucuronide cyclohexylamine salt (X-Gluc)), overnight at 37 °C. Stained tissues were washed several times in 70 % ethanol. All images of the GUS staining results were taken by a Nikon SMZ-U dissecting microscope equipped with a Nikon DXM 1200 CCD camera (Melville, NY, Nikon).

Germination assay

In order to ensure that differences in germination rates were not resultant from seed quality issues, wild-type and mutant plants were grown side by side under identical conditions in growth chamber conditions for seed production under long day conditions. Ripened seeds were dried for 1 month and were used for germination assays. As described above, sterilized seeds were stratified for 4 days at 4 °C in the dark. After stratification, seeds were shifted to 22 °C and

were monitored under continuous light conditions. Radicle emergence was used as the criterion for assessment of differences in germination between wild type and *atcsp1* mutant seeds. For germination assays with or without stratification, sterilized seeds were stratified or exposed to continuous light without stratification.

ABA ((±) Absciscic Acid, Sigma-Aldrich, St. Louis) was dissolved in absolute methanol to make a 100 mM as a stock solution. The desired final ABA concentrations in plates were prepared by the adding stock solution to cooled MS agar after autoclaving. For negative control plates, identical volumes of methanol were added to MS agar plates. Sterilization and stratification was performed as described above and seeds were grown under continuous light conditions under the same temperatures as described above. Germination studies were repeated three times and averages and standard deviations were calculated. Images of germinated seeds were observed under a Nikon SMZ-U dissecting microscope equipped with a Nikon DXM 1200 CCD camera (Melville, NY, Nikon).

Results

Characterization of AtCSP1 protein

AtCSP1 is a unique gene encoded by a single exon lacking any introns with a nucleotide sequence of 900 bp in length with a protein sequence of 299 amino acids. As predicted by Pfam software (<http://pfam.sanger.ac.uk/>; Figure 3-1A), the AtCSP1 protein encodes a well conserved N-terminal cold shock domain and seven C-terminal CCHC retroviral-like zinc finger motifs that are interspersed by eight glycine-rich regions (Figure 3-1A). Among the four AtCSPs in Arabidopsis, AtCSP1 encodes the largest protein followed by AtCSP3. Amino acid sequences of AtCSP1 and AtCSP3 exhibit high similarity of 73.3 % and identity of 63.8% (<http://www.ebi.ac.uk/Tools/emboss/align/>). Alignment of amino acid sequences indicated that their CSD regions are highly conserved among both of these genes. It is possible that AtCSP1 and AtCSP3 might have resulted from a gene duplication event since they are very similar in sequence and clusters of similar genes are present in both of their genomic loci (data not shown).

In order to understand if AtCSP3 and AtCSP1 affect each other's gene expression, I studied *AtCSP1* and *AtCSP3* transcript abundance in *atcsp3* and *atcsp1* loss of function mutants, respectively. Since the loss of function mutants are derived from different ecotype backgrounds,

I used different wild type *Arabidopsis* backgrounds for comparative analyses of transcript abundance. The loss of function mutants exhibited a complete loss of gene expression for their corresponding genes (Figure 3-1C). Interestingly, in the *atcsp3* loss of function mutant, *AtCSP1* transcript was accumulated relative to the Columbia wild type background (Figure 3-1C). However in the *atcsp1* (GT606) gene trap line, *AtCSP3* expression was not affected (Figure 3-1C). These results indicate that *AtCSP3* negatively affects the expression levels of *AtCSP1* *in planta* by an unknown mechanism.

Identification of a Gene Trap line (GT606) as an *atcsp1* loss of function mutant

To obtain an *atcsp1* loss of function mutant, I searched all of the *Arabidopsis* databases and identified a gene trap line named (GT606) utilizing the GUS gene as a reporter marker. I genotyped this line using the Ds5-1 primer, which recognizes the dissociation (Ds) insertion site, and gene specific primers for specific amplification of *AtCSP1*. The flanking Ds insertion was identified at +448 base pair downstream from the first initiation codon in the *AtCSP1* coding region (Figure 3-2A). I confirmed if this mutant generates a null transcript of *AtCSP1* by employing semi-quantitative RT-PCR analysis. As reported in the comparative CSP gene expression test, *AtCSP1* is expressed at a lower level relative to the three other *AtCSPs* (Nakaminami, 2009). *AtCSP1* was not detected prior to 35 cycles in semi quantitative RT-PCR analysis (Figure 3-2B, F1+R1). The GT606 gene trap line did not contain any full length transcript under the same cycling conditions. In contrast, a non-flanking region in 3'UTR was amplified clearly in both wild type and the GT606 gene trap line. Therefore, the GT606 mutant generates a truncated *AtCSP1* transcript, and likely renders it as a loss of function mutant due to alteration of coding region for a the *AtCSP1* gene locus.

GUS gene expression analysis

The GT606 gene trap line (*atcsp1*) used in our experiments contains a GUS gene insertion within the *AtCSP1* locus in the same orientation of the *AtCSP1* gene, resulting in the translational fusion of *AtCSP1* and GUS. I took advantage of this line for functional analysis of an insertional mutant and coupled it with expression analyses for temporal and spatial characterization of *AtCSP1* transcript in various tissues.

In early seedlings at 4 days after germination (DAG), cotyledons showed a high intensity of GUS staining (Figure 3-3A). Hypocotyls showed less GUS staining but embryonic root areas exhibited very high GUS staining. In the same seedlings, the primary root tips also showed high levels of GUS gene expression. Interestingly, the GUS gene expression in cotyledons decreased following their expansion and finally disappeared in cotyledons of 10 DAG plant (Figure 3-3B and C). At this stage, high GUS expression was observed in new rosette leaf primordia. However, the GUS expression was restricted to leaf base areas during leaf expansion and disappeared completely in mature leaves, as observed during cotyledon development (Figure 3-3C). A similar GUS staining pattern was observed in the leaves of middle aged *atcsp1* plants (Figure 3-3D). High GUS expression was observed in the tips of primary roots (Figure 3-3A). High GUS expression was also observed in lateral root primordia and preceded in apical regions during lateral root development. In roots, GUS expression is restricted to root tips only (Figure 3-5I). In addition, inflorescence meristems showed high levels of GUS expression (Figure 3-3E). GUS expression was also observed in shoot apical meristem of but expression was less in older stem tissues. At the base of auxiliary tissues such as cauline leaves and secondary stems, high levels of GUS expression were detected. In reproductive tissues, floral buds showed high GUS expression (Figure 3-3F), however only carpel tissues exhibited high GUS expression among the four different floral organs (Figure 3-3 F and G). During silique growth, GUS gene expression was observed in stigmas and the abscission zone (Figure 3-3G and H). However, in mature siliques, GUS expression was observed only at the abscission zone. Seeds were not stained at any stage of their development (Figure 3-4). In summary, by using gene trap line, I determined that *AtCSP1* is highly expressed in early developmental stages of various tissues and expression decreases gradually as the tissue matures.

Germination analysis of *atcsp1* and relationship with stratification

atcsp1 does not exhibit any atypical phenotypes in the morphology of vegetative and reproductive tissues (data not shown). I could not identify any delay in developmental phase transitions between respective tissues (data not shown). However, I determined that *atcsp1* seeds exhibit an early germination mutant phenotype (as early as 18 hours compared to 48 hours for wild type) after four days of stratification. Thus, *atcsp1* seeds germinated 30 hours earlier under normal continuous light conditions (Figure 3-4). Cotyledons of *atcsp1* also opened significantly

earlier than the wild type when compared to 42 hours after light exposure (HAL) (Figure 3-5 B). The initial germination, including all of the processes from testa rupture to radical protrusion, completed very quickly (in 6 hours) in *atcsp1*, while it took approximately 24 hours for completion in wild type seeds. Ultimately seeds from both wild type and *atcsp1* mutant plants showed 100 % germination. Germination in both *atcsp1* and wild type seeds was retarded without stratification treatment (Figure 3-4). Interestingly, the germination of *atcsp1* without stratification concluded at 30 HAL but only 96 % of the total seeds were germinated. Wild type *Ler* seeds also completed their germination without stratification but the germination was delayed relative to germination with stratification treatment. However, the germination timing of wild type seeds between without stratification and with stratification was not so different compared to that of *atcsp1*. Also, wild type *Ler* seed germinated at a rate of approximately 83 % without stratification while 98 % of *atcsp1* seeds germinated. These observations indicate that loss of function of *AtCSP1* promotes early germination and that *atcsp1* was more sensitive to stratification treatment compared to wild type *Ler*. However, total germinated seed number of *atcsp1* was not affected by treatment of stratification.

To monitor the *AtCSP1* transcript abundance during seed germination with or without stratification, we analyzed GUS expression in *atcsp1* grown under continuous light at 22 °C. The imbibed seeds that were treated with or without stratification did not show any GUS expression before testa rupture at 12 HAL (Figure 3-4C). During the radicle protrusion stage at 18 HAL, GUS was expressed in primary roots and accumulated around the radicle emergence area but not inside the seed. At 24 HAL, GUS expression was extended to cotyledons and the growing primary root. After 36 hours, GUS expression was concentrated in roots and newly generated hypocotyls. Opened cotyledons exhibited high expression of the GUS gene. In the case of germinated seeds without stratification, GUS expression was detected from the pre-germination stage (Figure 3-4C-24 HAL). However, GUS expression was restricted to the inside of the seed that was swollen by water uptake while radicles and hypocotyls did not show any GUS expression (Figure 3-4C-36 HAL, marked by circle). At 36 hours, GUS expression showed a similar pattern to that of germinated seeds that were pre-treated with stratification. After 36 hours, seedling growth speed was the same in un-stratified seeds and stratified seeds. In both cases, cotyledons were stained in identical areas (Figure 3-4C, 48 HAL). Therefore, GUS staining analysis of stratified *atcsp1* seeds during germination indicated that *AtCSP1* is expressed

highly in germinating seeds and seedling tissues like radicles, hypocotyls and cotyledons. In contrast, in the seeds that are not treated with stratification, *AtCSP1* gene expression was mainly observed inside of seeds and was not observed in seedling tissues. After the opening of cotyledons, GUS expression patterns were similar in both cases.

Relationship of ABA and germination in *atcsp1* mutants

ABA is a representative phytohormone which plays a role in seed germination and dormancy release. To understand if the early germination phenotype of *atcsp1* is related to defects in ABA synthesis, signaling or perception, I measured germination rates under various ABA concentrations. I counted the number of germinated seeds on MS plates containing 1 and 5 μ M ABA after a 4 day stratification period. On MS medium with 10 μ M ABA, most seeds failed to germinate (data not shown). On 1 μ M ABA plates, wild-type *Ler* seeds germination was delayed to 72 HAL. In contrast, however, seeds of *atcsp1* germinated completely at 36 HAL, which is an 18 hour delay compared to the normal germination timing in *atcsp1* seeds without ABA treatment (Figure 3-5 A). Under a 5 μ M ABA concentration, the negative impact on germination rates was more severe than that of the 1 μ M ABA concentration, however the germination of *atcsp1* was still higher than wild type. All of the seeds plated on MS medium with 5 μ M ABA were still germinating until 7 days after plating, but *atcsp1* concluded its germination earlier than wild type (Figure 3-5A).

The representative picture showing the differences in germination of *Ler* and *atcsp1* under various ABA concentrations was depicted in Figure 3-5 B. As shown in Figure 3-4, *atcsp1* germinated 30 hours earlier than wild type in the absence of ABA. In Figure 3-5B, cotyledons opened in the majority of *atcsp1* seeds at 42 HAL, while a portion of the wild type *Ler* seeds just initiated radicle protrusion at the same time point under treatment with 1 μ M ABA. These data indicate that *atcsp1* and wild type *Ler* are delayed in their germination by ABA treatment but the severity is less in *atcsp1* relative to wild type plants.

Discussion

AtCSPs have been characterized as nucleic acid binding proteins but their *in vivo* function in Arabidopsis is not clearly understood. Even though all AtCSPs appear to be expressed in all tissues, they show high levels of expression in tissues which exhibit cell

divisions and differentiation such as shoot apical meristems and developing siliques (Nakaminami 2009). In good accordance with the aforementioned gene expression pattern, RNAi mediated gene silencing of *AtCSP2* exhibited impairment in plant development (Fussaro 2007). Therefore, it is plausible that *AtCSPs* may have a similar functional role as other eukaryotic and bacterial cold shock domain proteins in relation to growth and development. Eukaryotic CSPs have been reported to function in tissues with actively dividing cells (Kohno 2003). LIN28, a CSP from *C. elegans* plays an important role in cell differentiation (Moss 2003). LIN28 regulates timing of development by influencing the translation and stability of mRNAs in P bodies during cell differentiation (Balzer 2007). Using a gene trap line with a GUS gene serving as a reporter, I show that *AtCSP1* is highly expressed in meristematic tissues such as shoot apices, inflorescences and root tips. Typically, *AtCSP1* is highly expressed in root and leaf primordia and as the tissues develop, *AtCSP1* expression is concentrated to tissue areas where cell differentiation occurs (Figure 3-3). During seed germination, this pattern of expression of *AtCSP1* was observed in seedling tissues like radicles, hypocotyls and cotyledons (Figure 3-4). However, *atcsp1* did not exhibit any abnormal tissue phenotypes such as alterations in the shape and size of the tissue. However, early germination was observed for *atcsp1* indicating that the *AtCSP1* gene may regulate the timing of tissue development in a similar manner as LIN28.

It was previously reported that *AtCSP1* exhibits nucleic acid binding activity. It was then later shown that the *AtCSP1* protein also has DNA melting activity and can promote RNase activity, implicating that it can function as an RNA chaperone to affect RNA degradation or translation (Kim 2007). In addition, the association of CSPs with other factors is important for their activity. For example, YB-1, which is a well known vertebrate CSP, associates with other nucleic acid binding proteins including YB-1 itself, to function in the regulation of gene transcription, translation, and RNA stability (Graudreault 2004, Izumi 2001). Similarly, *AtCSP1* may also function in association with other proteins and may play a functional role in the determination of tissue developmental timing.

Early germination is a typical phenotype of *atcsp1* (Figure 3-4). Germination is a complicated process which is affected by environmental regulators such as light, temperature, and water uptake. A balance exists between germination potential and the maintenance of dormancy to determine when germination occurs. Our observation of seed germination, with or without stratification, indicated that cold treatment did affect seed germination of *atcsp1* (Figure

3-4). When seeds were not stratified, *AtCSP1* expression was not detected in radicles. Interestingly, the radicle growing speed after the radicle protruded from the seed coat was consistent between *Ler* and *atcsp1* in both tests. In addition, growth of seedlings after germination was not altered in both cases. These data indicate that stratification with cold temperature mainly promotes embryo expansion which is followed by endosperm rupture and radicle protrusion. In fact, *AtCSP1* has been reported as cold responsive gene in seedlings (Kim 2004, Karlson 2003), therefore, it is possible that cold treatment could result in the accumulation of *AtCSP1*, which in turn has an effect on seed germination. I was unable to identify any seeds showing a strange structure or shape in *atcsp1* mutant seeds. I was also unable to definitively identify a process within the seeds which results in the early germination phenotype in *atcsp1*.

Reduced sensitivity of *atcsp1* to ABA indicates that phytohormone responsiveness is likely an important factor affecting the early germination phenotype of *atcsp1*. As mentioned before, ABA maintains seed dormancy which interferes with seed germination. Therefore, it is possible that *atcsp1* may be less sensitive to ABA, resulting in early germination. Taken together, *AtCSP1* plays a role in the determination of tissue developmental timing during germination. The early germination phenotype of *atcsp1* and the cold responsiveness of *AtCSP1* suggest that stratification may be a functionally important component for *AtCSP1* in regulating the timing of embryo expansion timing.

Figure 3-1. Conserved domain architecture of AtCSP1 and its redundancy with AtCSP3

A) Predicted domain architecture of the AtCSP1 protein. Amino acid sequences of AtCSP1 were predicted with Pfam web based software.

B) Clustal W alignment between AtCSP3 and AtCSP1

C) Semi-quantitative RT-PCR for comparison of *AtCSP1* and *AtCSP3* gene redundancy in loss of function mutants of AtCSP1 and AtCSP3. Arabidopsis Actin 1 (*AAC1*) was used as an internal standard.

Figure 3-2. Flanking position of the Gene Trap line GT606 (*atcsp1*) within the *AtCSP1* locus

A) Genetic map of the insertion site of the GT606 gene trap line in *AtCSP1*. White and black boxes represent the exon and UTR regions of *AtCSP1*, respectively. GT606 contained an insertion at +449 base pairs relative to the first start codon in the *AtCSP1* coding region. B) Semi-quantitative RT-PCR analysis of *AtCSP1* expression. PCR was performed for the described cycle numbers with the primers whose position on the *AtCSP1* locus were marked in panel A). *AAC1* was used as an internal control. The image shown in figure was chosen as a representative image from three replicate reactions.

Figure 3-3. GUS expression pattern within the GT606 (*atcsp1*) gene trap line

A) GUS expression during early seedling development of transgenic Arabidopsis. 4 DAG seedlings, B) 10 DAG seedlings, with emergence of 1st and 2nd rosette leaves C) Leaf primordia of 3rd rosette leaves in 12 DAG, D) 21 DAG plant, E) Inflorescence meristem from 28 DAG plant, F) Floral bud and early flower from 35 DAG plant, G) Open flower and early developed silique at 42 DAG, H) Fully matured silique and late open flower of 45 DAG plant, I) Detection of GUS staining during lateral root growth. From left to right, lateral root primordia, 1 and 2 days after primordia generation, respectively. A-E) GUS expression patterns were detected from seedlings grown on MS plate under long day conditions, and F-I) used seedlings grown in soil under long day conditions.

Figure 3-4. Seed germination with or without stratification

A) Germination rate of Ler and *atcsp1* with (+Str) or without (-Str). All data represent the mean $\pm$ standard for three independent germination tests (n=100). All seeds were stratified and were germinated under continuous light conditions. B) Images of germination results at different time point. Hours after light exposure (HAL) in the germination test with stratification (+Str) and 36 HAL were selected as typical time points which showed significant differences in germination between Ler and *atcsp1* in different stratification conditions.

C) GUS expression during seed germination with or without stratification.

Figure 3-5. Seed germination in different ABA concentration

A) Germination rates of *Ler* and *atcsp1* on MS plate with 0 (MS), 1, 5 uM ABA. All data represent the mean $\pm$ standard for three independent germination tests (n=100). Seeds were germinated on MS plates under continuous light conditions. B) Images of germination on different MS plates containing ABA concentrations at 42 HAL.

SUMMARY

The aim of this study was to understand the function and role of three different AtCSPs among the four present in *Arabidopsis*. AtCSP1, AtCSP3, and AtCSP4 are expressed ubiquitously but they tend to accumulate highly in meristems and reproductive tissues which undergo active live cell differentiation and proliferation. Using loss of function mutants and over expression plants, we determined that the functions of the three AtCSPs are very diverse.

Loss of function mutant of AtCSP3 exhibited short length and long width leaf blade along with short petiole. The leaf cell size and shape was also affected by the loss of AtCSP3. The gene expression of LNG1 which determines the leaf cell polarity to the leaf length direction on a leaf blade was regulated positively by AtCSP3. This regulation by AtCSP3 was further depended on leaf cell expansion timing which occurs after the leaf cell differentiation and cell proliferation. Based on the data, we determined that AtCSP3 functions as a specific regulator of leaf cell elongation to leaf length direction by affecting the transcript accumulation of leaf cell polarity factor genes.

Plants overexpressing AtCSP4 showed a short silique and defective seed phenotype. Overexpression of AtCSP4 affected the mRNA accumulations of endosperm development genes such as MEA and FIS2. Also, the transcript abundance of several genes of MADS box proteins were altered by overexpression of AtCSP4. Using T-DNA insertion lines of AtCSP4 and comparison of mRNA accumulation of AtCSP2 and AtCSP4 mutants, I determined that AtCSP4 functions redundantly with AtCSP1.

Gene trap lines of AtCSP1 showed GUS expression mostly concentrated at tissue primordia, implicating that AtCSP1 may function as regulator of tissue developmental timing. Loss of function of AtCSP1 showed early germination phenotype which was further dependent on stratification.

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CHAPTER 1

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