Overexpression of AtCSP4 affects late stages of embryo development in Arabidopsis

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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. 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) contains a well conserved cold shock domain (CSD) and glycine-rich motifs interspersed by two retroviral-like CCHC zinc fingers. AtCSP4 is expressed in all tissues but accumulates in reproductive tissues and those undergoing cell divisions. Overexpression of AtCSP4 reduces silique length and induces embryo lethality. Interestingly, a T-DNA insertion atcsp4 mutant does not exhibit any morphological abnormalities, suggesting that the related AtCSP2 gene is functionally redundant with AtCSP4. During silique development, AtCSP4 overexpression induced early browning and shrunk seed formation beginning with the late heart embryo stage. A 50% 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, MEA and FIS2 transcripts, which are involved in endosperm development, were affected by AtCSP4 overexpression. Additionally, AtCSP4 overexpression resulted in up-regulation of several MADS-box genes (AP1, CAL, AG, and SHP2) during early stages of silique development. Collectively, these data suggest that AtCSP4 plays an important role during the late stages of silique development by affecting the expression of several development-related genes.

Key words: Cold shock domain, embryo development, gene expression, silique development.

Introduction

Cold shock domain proteins (CSPs) are among the most conserved nucleic acid-binding proteins consisting of a small gene family in bacteria, animals, and plants. CSPs participate in various cellular functions, which are mediated by their characteristic ability to bind nucleic acids (Braun, 2000; Evdokimova et al., 2001; Kohno et al., 2003). CSPs are mainly implicated in growth and development in eukaryotes 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 the cytosol into the nucleus in response to specific physiological, environmental, and growth stimuli. YB-1 functions in transcription regulation, DNA repair, and mRNA splicing, thereby affecting the growth and development of organisms (Wilkinson and Shyu, 2001; Faustino and Cooper, 2003; Kohno et al., 2003; Raffetseder et al., 2003). Under normal conditions, YB-1 is localized in the cytoplasm and functions as an RNA chaperone in cytoplasmic ribonucleoproteins (RNPs) and mediates translational repression (Evdokimova et al., 2001, 2006; Bader and Vogt, 2005). Mouse YB-1 is expressed during embryogenesis, and a YB-1-deficient mutant exhibits an embryo-lethal phenotype (Lu et al., 2005). MSY4, an
additional mouse CSP, is also highly expressed during embryogenesis and can rescue a YB-1 loss-of-function mutant during early embryogenesis (Lu et al., 2006). Similar to animal CSPs, CSPs from plants, such as Arabidopsis and rice, have also been implicated in development (Nakaminami et al., 2006; Fusaro et al., 2007; Sasaki et al., 2007; Chaikam and Karlson, 2008; Park et al., 2009). Among the four Arabidopsis CSPs, AtCSP2 (AtGRP2/CSDP2; At4g38680) is characterized for its function in affecting flowering time and reproductive tissue development, including seed development (Fusaro et al., 2007). Further studies are necessary to understand fully the functions of AtCSPs in relation to plant reproductive development.

The physiology and genetics of the embryogenesis process are well understood (Goldberg et al., 1994; Jurgens, 1995, 2001; Laux et al., 2004; Park and Harada, 2008). 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 and Jurgens, 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 a review, see Laux et al., 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. Apical and basal embryo development is genetically regulated by WUSCHEL-RELATED HOMEOBOX 2 (WOX2) and WOX8 genes, respectively (Haecker et al., 2004). Gnomi/Embo3, Pin-formed 7 (PIN7), Pin1, Gurke, Fackel (FK), and Monopteros (MP) genes also regulate apical–basal embryo formation (Torres-Ruiz et al., 1996; Galweiler et al., 1998; Schrick et al., 2000; Geldner et al., 2001, 2003; Aida et al., 2002; Friml et al., 2003).

Post-embryonic development is controlled by the LEAFY COTYLEDON (LEC) class of genes including LEC1, LEC2, and FUSCA3 genes (Parcy et al., 1997; Lotan et al., 1998; Luerssen et al., 1998; Stone et al., 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 (Kohler and Makarevich, 2006). The FIS family Polycomb group (PcG) proteins include: MEDEA (MEAFISI) ( Grossniklaus et al., 1998; Kiyosue et al., 1999), Fertilization Independent Seed 2 (FIS2) (Luo et al., 1999, 2000), Fertilization Independent Endosperm 3 (FIE/ FIS3) (Ohad et al., 1999), and MULTI-COPY SUPPRESSOR OF IRA1 (MSII) (Kohler et al., 2003a; Guitton et al., 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 (Ohad et al., 1996; Chaudhury et al., 1997, 2001; Grossniklaus et al., 1998; Chaudhury and Berger, 2001).

MADS-box proteins have been extensively studied in relation to their functional role in floral organ identity determination and transition of the floral meristem. MADS-box genes included in the ABC model are highly expressed during silique development and embryogenesis (Alvarez-Buylla et al., 2000; Parenicova et al., 2003; de Folter et al., 2004; Lehti-Shiu et al., 2005). Gene expression of AG, which is a carpel development gene, increases particularly again during fruit development and ripening (Seymour et al., 2008). Also, SHATTERPROOF (SHP) and FRUIT-FULL (FUL) are highly expressed and are functionally important during silique development (Gu et al., 1998; Ferrandiz et al., 2000; Liljegren et al., 2000; de Folter et al., 2004). 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 et al., 2005), especially AGAMOUS like-15 (AGL15) and AGL18 which are both expressed in endosperm and embryo tissue (Heck et al., 1995; Alvarez-Buylla et al., 2000). Constitutive overexpression of AGL15 in Arabidopsis delays leaf and flower senescence, as well as fruit maturation (Fernandez et al., 2000). AGL15 was recently shown to bind AtCSP2 and AtCSP4 promoters using a chromatin immunoprecipitation (ChIP) assay. These data support the hypothesis that AtCSP genes are regulated by MADS-box proteins and suggest that AtCSPs may function during silique development (Nakaminami et al., 2009).

In the present study, morphological analyses were performed in T-DNA insertion and gain-of-function AtCSP4 (AtGRP2b; At2g21060) overexpression 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 is altered in AtCSP4 overexpression lines. These data suggest that AtCSP4 alters silique development and embryogenesis by affecting genes involved in silique and seed development.

### Materials and methods

**Plant materials and growth conditions**

An AtCSP4 T-DNA insertion mutant 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, USA). Seeds were stratified at 4 °C for 4 d under dark conditions. All plants were grown in Metromix 360 soil (Scott Co., Marysville, OH, USA) under long-day conditions at 23 °C (16 h/8 h for light/dark cycle). To generate AtCSP4 overexpression lines, the coding region of AtCSP4 was amplified with gene-specific primers from a Col-0 wild-type plant with KOD Hot Start high fidelity Taq polymerase (Novagen, Gibbstown, NJ, USA) with the following primers: forward primer 5′-CAC CAT GAG CAG AGG AGG AGA CGT GAA C-3′; reverse primer 5′-ACG AGC ACC ACC GCT AGT GCA ATC CCT TGC-3′. The amplified coding sequence of AtCSP4 was cloned into the pENTR entry vector plasmid (Invitrogen, Carlsbad, CA, USA) according to...
Gene expression analysis
For characterizing gene expression in the actcsp4 T-DNA insertion mutant and 35S:NTAP:AtCSP4 overexpression lines, total RNA was extracted from leaf tissue using TRIzol® reagent (Invitrogen). For studying the expression of MADS-box and embryogenesis-related genes, total RNA was isolated from different tissues using Plant RNA Extraction Reagent (Invitrogen). cDNA was synthesized using 50 ng of total RNA with the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA). Semi-quantitative RT-PCR analysis was performed with the Go-Taq Flexi PCR kit (Promega, Madison, WI, USA) with the following thermocycling conditions: 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, for a specified number of cycles (as described within individual figures) with a final extension step for 7 min at 72 °C. Primers are listed in Supplementary Tables S1 and S2 available at JXB online.

For AtCSP4 quantification of transcripts within different tissues, Taq-Man probe quantitative real-time PCR (qRT-PCR) analysis with a TaqMan® Universal PCR Master mix (Applied Biosystems, Foster City, CA, USA) was utilized. Primers and TaqMan® probes for AtCSP4 and ACT2 genes were purchased from Applied Biosystems, and primer sequences are described in Supplementary Table S3 at JXB online. Thermocycling conditions were as follows: 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. With the exception of leaves, total RNA from different tissues was extracted with the Plant RNA extraction Reagent (Invitrogen) and cDNA was prepared as described above.

β-Glucuronidase (GUS) expression plant and histological analysis
To generate a GUS expression gene construct fused to the AtCSP4 promoter, a 2 kb upstream fragment of the AtCSP4 gene was amplified with KOD Hot Start DNA polymerase (Applied Biosystems). The following primers were designed, and incorporated Kpnl and NcoI restriction sites: forward 5′-TCTGGTACGAGCACCACCGCTAGC-3′ and reverse 5′-TCTGCTGGAAACCTCGGACGG-3′. The amplified DNA fragment was digested by restriction enzymes and subsequently ligated into a pre-digested pCAMBIA1303 binary vector in-frame with the GUS gene. This vector was transformed into the LBA4404 Agrobacterium strain with a MicroPulser electroporator as previously described. Transformation of this vector into Col-0 wild-type Arabidopsis was performed by the floral dip method (Clough and Bent, 1998). Transgenic Arabidopsis were screened on 1× MS containing 1% sucrose, 1% phytoagcar, and 25 µg ml⁻¹ gentamycin (Caisson Labs, North Logan, UT, USA).

Subcellular localization of AtCSP4
The coding region of the AtCSP4 gene was amplified with the following primers: 5′-TCTGCTGGAAACCTCGGACGG-3′ and 5′-AGACCATGTTACGAGCACCACCCGTCAGACGTC-3′. The amplified PCR products were digested with these restriction enzymes and ligated into a pre-digested synthetic GFP(s65T) plasmid. Sequence integrity and maintenance of the reading frame were confirmed by DNA sequencing. A 5 µg aliquot of sGFP empty plasmid or sGFP-AtCSP4 plasmids was coated onto DM-10 Tungsten particles according to the manufacturer’s instructions (BioRad). A Biologic® PDS-1000 particle bombardment system was used for transient transformation of onion cells by employing 25 inches of Hg vacuum, a 1000 psi rupture disc, and a 12 cm target distance (BioRad). Bombarded onion epidermal cell layers were incubated at 22 °C overnight under dark conditions. DIC and GFP images were obtained by a Zeiss Axiosmager LSM-510 confocal microscope and analysed with LSM image analysis software (Carl Zeiss AG, Germany).

Results
Characterization of the AtCSP4 gene
AtCSP4 (AtGRP2b; Atg21060) and AtCSP2 (AtGRP2/CSDP2: Atg438680) are highly homologous to one another and encode smaller sized proteins relative to the other two Arabidopsis CSPs (AtCSP3; Atg17870 and AtCSP1/CSDP1:Atg36020). The AtCSP4 gene encodes a 603 bp transcript which lacks introns. 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 (Supplementary Fig. S1A at JXB online). AtCSP4 shows 86% amino acid homology to AtGRP2 and their CSD regions are highly conserved (Supplementary Fig. S1B). 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, AtCSP2 transcript levels were studied in a T-DNA insertion mutant and AtCSP4 overexpression plants (Supplementary Fig. S1C). As expected, the T-DNA insertion mutant and overexpression plants showed a loss and accumulation of AtCSP4 transcript, respectively (Supplementary Fig. S1C). In order to assess if AtCSP4 levels affect AtCSP2
transcript accumulation, experiments were also carried out
to test for alterations of AtCSP2 transcript abundance, and
none were detected. These data indicate that AtCSP4 and
AtCSP2 do not participate in any feedback regulation of
one another.

AtGRP4 expression analyses

The Genevestigator (http://www.genevestigator.ethz.ch/)
public microarray database confirmed that 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 AtCSP genes (Nakaminami et al., 2009).
Comparative analysis of AtCSP4 expression using micro-
array and real-time PCR data confirmed that the AtCSP4
transcript is highly abundant in reproductive tissues,
especially in carpels and siliques. To characterize the tissue
specificity of the AtCSP4 transcript, a Taq-Man probe qRT-PCR assay was employed. As shown in Fig. 1A, the
AtCSP4 mRNA is expressed in all tissues similar to the pat-
tern of AtCSP2/AtGRP2 (Fusaro et al., 2007; Nakaminami
et al., 2009). 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. In particular, AtCSP4 is highly abundant in
mature siliques harbouring full-grown seeds.

To monitor the tissue-specific gene expression of AtCSP4
in planta, transgenic plants were developed by transforming
wild-type plants with the T-DNA binary vector containing
the AtCSP4 promoter fused to the GUS reporter gene
(Supplementary Fig. S2A at JXB online). T2 generation
transgenic plants were used for GUS expression assays.
GUS staining results revealed that AtCSP4 is expressed in
all tissues in good accordance with data obtained by qRT-
PCR analyses. 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. At 21 days after germination (DAG), lateral root
tips contained high levels of GUS (Fig. 1D). In young
seedlings at 7 DAG, the hypocotyl contained strong GUS
gene expression relative to other tissues (Fig. 1B). Tissues
exhibiting high numbers of cell divisions such as young
leaves, leaf primordia, and shoot apices showed high GUS
gene expression (Fig. 1C). In leaf blades, GUS expression
was particularly 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 exhibited GUS expression in young
leaves, but its expression disappeared gradually following
leaf expansion. The apical meristem exhibits high activity of
the AtCSP4 promoter, resulting in dark blue staining in the
centre of shoot apices from both primary and lateral shoots
(Fig. 1C) and the inflorescence meristem (Fig. 1E, F).
Reproductive tissues exhibit stronger AtCSP4 promoter
activity relative to vegetative tissues (Fig. 1F–H). Siliques
with mature seeds showed the highest level of GUS gene
expression relative to any other tissue (Fig. 1H). Specifi-
cally, the dehiscence area and two valves in siliques stained
very strongly (Fig. 1H). Taken together, the qRT-PCR and
GUS staining results indicate that AtCSP4 is expressed in
multiple tissues but accumulates preferentially in meriste-
matic and reproductive tissues.
Morphological analysis of an AtCSP4 overexpression and a T-DNA insertion mutant

To characterize AtCSP4 gene function in planta, an AtCSP4 overexpression and a T-DNA insertion mutant line was functionally characterized. The T-DNA insertion line was obtained from the GABI-kat 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, it was confirmed that the T-DNA insertion occurs within the cold shock domain-coding region at +91 bases from the first initiation codon (Fig. 2A). When total RNA was isolated and converted into cDNA, AtCSP4 transcript was not detected in GK-623B08.01 with a primer pair that was designed to amplify full-length AtCSP4 (Fig. 2B: F1+R1), indicating disruption of the AtCSP4 locus.

An overexpression line of AtCSP4 fused to an N-terminal TAP-tag that was driven by a 35S promoter (35S:NTAP:AtCSP4) was generated as a means to observe the functional effect of ectopic overexpression of AtCSP4 (Supplementary Fig. S2B at JXB online). Seventeen independent gentamycin-resistant lines were isolated from the T1 generation. Four representative lines were characterized in greater detail, with all exhibiting embryo-lethal and abortive phenotypes (Fig. 3). AtCSP4 transcript abundance was visualized with semi-quantitative RT-PCR, confirming elevated levels of AtCSP4 transcript in overexpression lines relative to the wild type (Fig. 2C).

To gain insight into the function of AtCSP4, the phenotypes of T-DNA insertion mutant and overexpression lines of AtCSP4 were compared with the wild type under long-day light conditions. As shown in Fig. 3, the 35S:NTAP:AtCSP4 overexpression lines exhibit typical phenotypes in mature seedlings. 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 (Fig. 2D). In addition, total leaf number (Fig. 2E) and leaf size (not shown) did not differ significantly from wild type. With respect to flowering time, all lines initiated flowering at 23 DAG, similar to wild-type plants (data not shown). Four independent transgenic plants harbouring the 35S:NTAP empty vector were also characterized and did not exhibit any phenological or growth abnormalities (data not shown). Collectively, these data demonstrate that overexpression or disruption of AtCSP4 does not affect vegetative development or flowering time.

Although atypical phenotypes were not observed during vegetative growth, brown and shrunken seeds were observed within mature siliques of 35S:NTAP:AtCSP4 overexpression lines. It was not possible 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 strongly support the hypothesis that the embryo-lethal phenotype arrests normal seed development in 35S:NTAP:AtCSP4 homozygous lines.

Reproductive tissue morphology of the wild type, the homozygous atcsp4 T-DNA insertion mutant, and the representative heterozygous 35S:NTAP:AtCSP4-3 overexpression line was compared for all tests. The distance between individual siliques on the primary stem of atcsp4 and 35S:NTAP:AtCSP4 plants was similar to that of the wild type (Fig. 3A), and the size and structures of atcsp4 and 35S:NTAP:AtCSP4 flowers were not altered (Fig. 3B).

However, the length of mature siliques in the 35S:NTAP:AtCSP4-3 line was shorter than that of the wild-type and atcsp4 (Fig. 3C, D). Overexpression of the NTAP:AtCSP4 fusion protein resulted in an abortive or embryo-lethal phenotype (Fig. 3F).

The extent of embryo lethality varied among the four characterized lines. Specifically, the 35S:NTAP:AtCSP4-12 line possessed white immature seeds as well as embryo-lethal seeds. 35S:NTAP:AtCSP4-3 and 35S:NTAP:AtCSP4-8 lines showed a very similar distribution of embryo-lethal seeds and the 35S:NTAP:AtCSP4-1 line contained the lowest number of embryo-lethal seeds. The proportion of the 35S:NTAP:AtCSP4-3 plants generating short siliques harbouring embryo-defective seed to the plants generating wild-type siliques and seeds is 55%/45% in T3 plants that were grown in soil without antibiotic selection (n=36). The numbers of defective seeds and normal wild-type seeds in short siliques were also counted 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%/49% (Table 1). However, the atcsp4 T-DNA insertion mutant exhibited typical siliques morphology and contained mature seeds. Taken together, these data confirm that the AtCSP4 gene alone is not an essential gene for completion of the reproductive stage. This hypothesis is supported further by the fact that AtCSP4 is 1000-fold down-regulated in the Ler ecotype relative to Col-0 (Nakaminami et al., 2009). However, ectopic overexpression of AtCSP4 induces short siliques that harbour ~50% embryo-defective seeds. Overexpression of AtCSP4 disrupts normal seed and siliques development via an unknown functional mechanism.

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

<table>
<thead>
<tr>
<th>Plant genotype</th>
<th>Defective seeds</th>
<th>Wild-type seed</th>
<th>Total no. of seeds analysed</th>
<th>No. of siliques analysed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0</td>
<td>0.58%</td>
<td>99.42%</td>
<td>868</td>
<td>20</td>
</tr>
<tr>
<td>atcsp4</td>
<td>0.69%</td>
<td>99.31%</td>
<td>874</td>
<td>20</td>
</tr>
<tr>
<td>35S:NTAP:AtCSP4-3</td>
<td>51.66%</td>
<td>49.34%</td>
<td>2228</td>
<td>50</td>
</tr>
</tbody>
</table>
overexpression of AtCSP4. Seeds were cleared and extracted from early maturing stages of siliques derived from 35S:NTAP:AtCSP4-3, atcsp4 T-DNA insertion mutant, and wild-type plants. Each silique stage was referred to according to the stages of embryo development as described in the AtGenExpress website (http://www.genomforschung.uni-bielefeld.de/GF-research/AtGenExpress-SeedSiliques.html). As shown in Fig. 4F–J, 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 exhibits two different seed phenotypes (embryo-lethal and wild-type) within the same silique, all seeds were carefully observed until the embryo-defective phenotype appeared. No abnormalities were observed in seeds from 35S:NTAP:AtCSP4-3 plants until after the early heart stage (as seen in Fig. 4K and L). After the heart stage, 35S:NTAP:AtCSP4-3 seeds did not develop to the torpedo stage and the endosperm began shrinking and the seed coat rapidly turned brown (Fig. 4M–O). In contrast, seeds from the atcsp4 T-DNA insertion mutant and wild type maintained a green colour and normal shape (Fig. 4C–E, H–J). Wild-type-appearing seeds in 35S:NTAP:AtCSP4-3 plants continued developing normally and did not differ morphologically from Wild type (data not shown). Thus, it is concluded that the embryo-lethal mutation in homozygous 35S:NTAP:AtCSP4-overexpressing seeds results from defects occurring during the late heart stage of embryo development.

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

To elucidate the relationship between defective seed maturation and related gene expression in the 35S:NTAP:AtCSP4-3 line, the gene expression levels of both initial floral organogenesis- and seed development-related genes were studied in 35S:NTAP:AtCSP4 plants. As shown in Fig. 4M, embryo development in 35S:NTAP:AtCSP4-3 is impaired from the late heart stage. Publicly available microarray data of silique development revealed that...
AtCSP4 peaks in gene expression in the carpel developmental stage, which occurs during early silique development (http://www.genevestigator.ethz.ch/). Therefore, our initial focus was on gene expression of candidate genes controlling early stages of silique development.

MADS-box proteins have been functionally implicated in early silique development, ovule development, and seed embryogenesis (Lehti-Shiu et al., 2005). Since one of the characteristic mutant phenotypes of 35S:NTAP:AtCSP4 plants was a shortened silique length, the expression of MADS-box protein genes was monitored in developing flowers and two stages of silique development. Additionally, the transcript expression of pistil and fruit development genes such as FUL and SHP2 was also determined. In floral buds and stage 1 siliques, transcripts of AP1, CAL, FUL, and AG exhibited slight accumulation in 35S:NT AP:AtCSP4-3 plants relative to the wild type. The expression pattern for SHP2 exhibited the most noticeable shift, with accumulation occurring earlier in overexpression plants. With the exception of SHP2, gene expression patterns were similar between wild-type and overexpression plants (Fig. 5A). In summary, overexpression of AtCSP4 predominantly affects the mRNA accumulation of SHP2 during floral and silique development with minimal effect on AP1, CAL, AG, and FUL. Shortened siliques in overexpression plants may result from the collective alterations in gene expression patterns for several MADS-box protein genes at the onset of the early stages of silique development.

The relationship between the impairment of silique development (shortened size) and the development of abnormal seeds was also studied by comparison of MEA, FIS2, and FIE transcripts. Transcripts for FUS3 and LEC1 genes were not altered in overexpression plants relative to wild type (Fig. 5B). The ABI3 transcripts could not be

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**Fig. 3.** 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=2 cm). (B) Comparison of flower size. Opened flowers were collected from primary bolts at 35 DAG (bar=0.2 cm). (C) Comparison of silique size. Fully matured siliques were detached at 49 DAG (bar=1 cm). (D) Silique size comparison. Siliques sizes were determined with the longest siliques from 50 different plants from wild-type, atcsp4, and 35S:NTAP:AtCSP4-3 plants (n=50). (E) Seed number comparison. Seeds were counted from the same siliques which were used in D. All plots in D and E show means ± SD. (F) Seed formation and maturation of the wild type and atcsp4, and the T3 generation of 35S:NTAP:AtCSP4-3, 1, 8, and 12 lines. All siliques were opened from fully matured siliques at 49 DAG (bar=0.5 cm).
detected in the 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 (Fig. 5B). Consistent with previously reported data, the MEA transcript was detected in inflorescences and open flowers before fertilization and it disappeared as embryo development proceeded in wild-type plants (Baroux et al., 2006). In contrast, the MEA transcript in 35S:NTAP:AtCSP4-3 was expressed at a much greater level relative to that of the wild type in the later stage of embryo development (Fig. 5B). Two other endosperm developmental genes, FIS2 and FIE, were expressed at similar levels in floral buds in wild-type and 35S:NTAP:AtCSP4-3 plants, but FIS2 expression was reduced during stage 3 in 35S:NTAP:AtCSP4-3 plants. In summary, expression of MEA and FIS2 is affected during the floral bud and early stages of silique development in 35S:NTAP:AtCSP4-3 plants. MEA transcripts were most notably affected during stage 3 of silique development. Expression of LEC1, FUS3, and FIE was not affected by overexpression of NTAP:AtCSP4, indicating that these genes are not likely to contribute to the defective seed phenotype observed in 35S:NTAP:AtCSP4-3 plants.

Subcellular localization of AtCSP4 in onion cells

The subcellular localization of AtCSP4 was examined by making a C-terminal fusion to sGFP(s65T) (Supplementary Fig. S2C at JXB online). The 35S:sGFP and 35S:AtCSP3:sGFP plasmids were transformed into onion epidermal cells by particle bombardment. 35S:sGFP was expressed in both the nucleus and cytosol (Fig. 6), and 35S:AtCSP4:sGFP-transformed onion cells also exhibited a nuclear and cytoplasmic localization.

Discussion

Putative redundant function of AtCSP4/AtGRP2b and AtCSP2/AtGRP2

CSPs are commonly found in plants (Karlson and Imai, 2003); however, despite recent functional studies in wheat, Arabidopsis, and rice, their precise functional roles remain poorly understood (Karlson et al., 2002; Karlson and Imai, 2003; Nakaminami et al., 2006; Fusaro et al., 2007; Sasaki et al., 2007; Chaikam and Karlson, 2008; Kim et al., 2009; Nakaminami et al., 2009). Arabidopsis possesses four CSPs which can be sorted into two groups based on similarity of amino acid sequence and motifs. AtCSP2/AtGRP2/CSDP2 and AtCSP4/AtGRP2b 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 (Supplementary Fig. S1B at JXB online). Detailed tissue-specific expression analyses confirmed that both of these genes are highly expressed in meristemic and reproductive tissues (Nakaminami et al., 2009). Although the AtCSP4 transcript is less abundant than AtCSP2, AtCSP4 exhibits a similar trend of expression during development (Nakaminami et al., 2009).
et al., 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 (Nakaminami et al., 2009). 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 (Fig. 2). Interestingly, when $AtCSP4$ is overexpressed, severe developmental effects occur. $AtCSP2$ transcript is unaffected in $atcsp4$ knockout and overexpression mutant lines. The functional and interactive relationship between $AtCSP4$ and $AtCSP2$ is not known at this time.

qRT-PCR analyses (Fig. 1A) (Nakaminami et al., 2009) and GUS reporter data confirm that $AtCSP4$ is predominantly expressed in meristematic and reproductive tissues, especially in siliques (Fig. 1B–H). These GUS data are also in good agreement with $AtCSP2$ gene expression data previously obtained by in situ hybridization and GUS analysis (Fusaro et al., 2007; Sasaki et al., 2007; Nakaminami et al., 2009). When a homozygous $atcsp4$ T-DNA insertion mutant was characterized, no atypical phenotypes were observed. These data are in contrast to those previously observed for RNA interference (RNAi) mutant lines of $AtCSP2$ (Fusaro et al., 2007). Taken together with these aforementioned findings from RNAi and the confirmed 1000-fold reduction of AtCSP4 in the Ler ecotype, the lack of any aberrant phenotype in vegetative and reproductive tissues in the $atcsp4$ T-DNA insertion mutant (Figs 2–4), it is hypothesized that $AtCSP2$ has greater functional importance in planta (Fusaro et al., 2007; Sasaki et al., 2007; Nakaminami et al., 2009). Furthermore, the similarities in the expression patterns of $AtCSP4$ and $AtCSP2$ suggest $AtCSP4$ and $AtCSP2$ are functionally redundant.

**Overexpression of AtCSP4 reduces siliquae lengths and induces embryo lethality**

In an effort to understand in planta functional roles for $AtCSP4$, an $atcsp4$ homozygous T-DNA insertion mutant and four independent $35S:NTAP:AtCSP4$ overexpression lines were characterized. The $atcsp4$ T-DNA insertion mutant does not exhibit any atypical morphological or developmental phenotypes (Figs 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, an RNAi mutant of $AtCSP2$ showed early flowering and abnormal flower generation (Fusaro et al., 2007). On the other hand, overexpression of $AtCSP4$ resulted in atypical phenotypes in reproductive tissues such as shortened siliquae size and defective seed maturation. However, the developmental timing of vegetative and reproductive tissues was not affected by ectopic overexpression of $AtCSP4$ (Fig. 2). In heterozygous $35S:NTAP:AtCSP4-3$ plants, mature siliques contained ~50% defective seeds (Table 1), resembling a FIS gene mutant with impaired endosperm development (Grossniklaus et al., 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 a similar ratio of defective seed (Kohler et al., 2003a, b). Embryo formation in $35S:NTAP:AtCSP4-3$ arrests at the late heart stage (Fig. 4) which is the same time point where defects in embryo formation are observed for a $fis$ mutant allele (Ohad et al., 1996; Chaudhury et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999). The defective seed formation in $AtCSP4$ overexpression lines probably results in the late heart stage (Fig. 4) which is the same time point where defects in embryo formation are observed for a $fis$ mutant allele (Ohad et al., 1996; Chaudhury et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999). The defective seed formation in $AtCSP4$ overexpression lines probably results

**Fig. 5.** Semi-quantitative RT-PCR analysis of genes involved in siliquae development and seed embryogenesis. (A) Semi-quantitative RT-PCR analysis of MADS-box genes. $AP1$, $CAL$, $AG$, and $SHP2$ were used as marker genes related to endosperm development. $MEA$, $FIS2$, and $FIE$ were used as maker genes related to endosperm development. Overexpression of $AtCSP4$ affects endosperm development genes such as $MEA$ and $FIS2$ but not $FIE$. Arabic numbers denote the siliquae stages. $AAC1$ was used as an internal control. All semi-quantitative RT-PCR figures are representative images from three replicate reactions.
from a parent-of-origin effect on the endosperm development process.

Expression of MADS-box and seed embryogenesis-related genes is altered by overexpression of AtCSP4

Microscopic observation of developing embryos in 35S:NTAP:AtCSP4-3 seeds revealed that early shrinkage of endosperm tissue initiates at the late heart stage (Fig. 4M). It is important to note that animal CSPs have also been implicated in embryo development. For example, mouse YB-1 and MSY4 proteins are highly expressed during embryogenesis, and a double knockout in mice cells prevent cell senescence, suggesting both genes share function in proliferative tissue in higher vertebrates (Lu et al., 2006). In Arabidopsis, ChIP analysis confirmed that AGL15, a well-studied MADS-box protein expressed in developing embryos, binds to the promoter regions of AtCSP4 and AtCSP2 (Nakaminami et al., 2009). These correlative data suggest that AtCSPs may also play an important role in silique development similar to their animal counterparts (Nakaminami et al., 2009). Therefore, it is of interest to further understand the functional relationship of plant CSPs during silique development.

MADS-box proteins commonly function in relation to floral transition, fruit development, and ovule development (Lehti-Shiu et al., 2005; Seymour et al., 2008). Silique development is established from carpel identity determination, which is regulated by AG, AP1, and FUL function redundantly in floral meristem identity determination but their function is divergent in relation to lateral development involving fruit development (Fernandez et al., 2000). The ful mutant generates a very small silique size due to defects in silique valve cell differentiation (Ferrandiz et al., 2000). Also, FUL interferes with SHP1 and SHP2 gene expression, but AG induces their gene expression in early stages of silique development. Overexpression of AtCSP4 resulted in slight up-regulation of AP1, CAL, AG, and FUL in floral bud and silique tissues. SHP2 expression exhibited the greatest shift due to AtCSP4 overexpression.

As shown in Fig. 4, early embryo formation and endosperm development in 35S:NTAP:AtCSP4-3 is not retarded before the late heart embryo formation stage. However, its development ceased from the late heart stage of embryo formation (Fig. 4M). In 35S:NTAP:AtCSP4-3, transcripts of early embryo formation genes such as FUS3 and ABI3 could not be detected, while the expression of MEA and FIS2 that are involved in endosperm development was 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 MSII form a huge complex (~650 kDa) which may combine with FIS2 (Kohler et al., 2003b; Chanvivattana et al., 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 proteins in Arabidopsis, is increased after fertilization in the AtCSP4 overexpression line (Fig. 5).

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 et al., 2006a, b). 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. Judging from the previously published phenotype of various MADS-box protein mutants, it is presumed that overexpression of AP1, AG, and SHP2 does not result in embryo lethality (Mizukami and Ma, 1992; Mandel and Yanofsky, 1995; Liljegren et al., 2000).

The direct functional link between the ectopic over-expression of AtCSP4 and MEA is not known at this time. In animals, CSPs function in diverse processes at both the transcriptional and post-transcriptional level, such as transcriptional activation/repression, alternative splicing, RNA stability, and RNA masking, among others. In plants, the most well characterized CSP is NAB1, which functions in RNA masking of LHCBM mRNA at the post-transcriptional level (Mussgnug et al., 2005). Other plant CSPs have been suggested to function as RNA-binding proteins based on

![35S:sGFP](image1.png) ![35S:AtCSP4:sGFP](image2.png)

Fig. 6. AtCSP4 subcellular localization in onion epidermal cells. Transient analysis of the subcellular localization of AtCSP4 in onion epidermal cells (bar=125 μm). Similar to the 35S:GFP control, note that AtCSP4 localized to the cytosol and the nuclei.
the results of nucleic acid binding assays in wheat and *Arabidopsis* (Fusaro et al., 2007; Sasaki et al., 2007; Nakaminami et al., 2009). In the case of winter wheat WCS1P and *Arabidopsis* AtCSP1 (Nakaminami et al., 2006; Kim et al., 2007), RNA chaperone activity has been proposed on the basis of *in vivo* and *in vitro* assays; however, no data have yet indicated direct functions as transcriptional activators or repressors. Subcellular localization data revealed that AtCSP4 is a nucleocytoplasmic protein (Fig. 6), providing correlative evidence which suggests that AtCSP4 does not function directly to regulate transcription of target genes but rather exerts a functional effect via post-transcriptional regulation. Taken together with morphological and semi-quantitative RT-PCR analyses, shortened silique and embryo lethality may be the result of an independent alteration of gene transcripts for MADS-box protein and endosperm development-related genes in *AtCSP4* overexpression mutants.

**Supplementary data**

Supplementary data are available at *JXB* online.  
Figure S1. Secondary protein structure of AtCSP4 and redundancy with AtCSP2.  
Figure S2. Schematic representation for *AtCSP4* gene functional analysis.  
Table S1. Primer list for *AtCSP4* transcript detection and amplicon length of the PCR product with the R1 primer.  
Table S2. Primer list for semi-quantitative RT-PCR analysis.  
Table S3. List of context sequence and amplicon lengths for Taq-Man probes.

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**References**


