Arabidopsis Carboxyl-Terminal Domain Phosphatase-Like Isoforms Share Common Catalytic and Interaction Domains But Have Distinct in Planta Functions[1[W]]

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An Arabidopsis (*Arabidopsis thaliana*) multigene family (predicted to be more than 20 members) encodes plant C-terminal domain (CTD) phosphatases that dephosphorylate Ser residues in tandem heptad repeat sequences of the RNA polymerase II C terminus. CTD phosphatase-like (CPL) isoforms 1 and 3 are regulators of osmotic stress and abscisic acid (ABA) signaling. Evidence presented herein indicates that CPL3 and CPL4 are homologs of a prototype CTD phosphatase, FCP1 (TFIIF-interacting CTD-phosphatase). CPL3 and CPL4 contain catalytic FCP1 homology and breast cancer 1 C terminus (BRCT) domains. Recombinant CPL3 and CPL4 interact with AtRAP74, an Arabidopsis ortholog of a FCP1-interacting TFIIF subunit. A CPL3 or CPL4 C-terminal fragment that contains the BRCT domain mediates molecular interaction with AtRAP74. Consistent with their predicted roles in transcriptional regulation, green fluorescent protein fusion proteins of CPL3, CPL4, and RAP74 all localize to the nucleus. *cpl3* mutations that eliminate the BRCT or FCP1 homology domain cause ABA hyperactivation of the stress-inducible *RD29a* promoter, whereas RNAi suppression of *CPL4* results in dwarfism and reduced seedling growth. These results indicate CPL3 and CPL4 are a paralogous pair of general transcription regulators with similar biochemical properties, but are required for the distinct developmental and environmental responses. CPL4 is necessary for normal plant growth and thus most orthologous to fungal and metazoan FCP1, whereas CPL3 is an isoform that specifically facilitates ABA signaling.

Transcriptional induction of genes that encode stress tolerance determinants is an integral component of plant adaptation responses to adverse environments (Fowler and Thomashow, 2002; Seki et al., 2002; Maruyama et al., 2004). *Responsive to Dehydration* (*RD*) genes are ubiquitous outputs of plant stress signal pathway integration that is activated by low temperature, hyperosmolarity, and the plant hormone abscisic acid (ABA), and are presumed necessary for adaptation (Hasegawa et al., 2000; Viswanathan and Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). Promoters of genes comprising the *RD* transcriptome contain dehydration/cold-responsive and ABA-responsive elements that function with DREB/CBF and AREB/ABF DNA-binding transcription factors, respectively, in signal pathway-mediated activation (Stockinger et al., 1997; Liu et al., 1998; Choi et al., 1999; Uno et al., 2000; Kang et al., 2002). Gain- and loss-of-function studies have established that regulation of the *RD* transcriptome by these transcription factors is a primary determinant of desiccation and cold tolerance (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Chinnusamy et al., 2003).

Recent studies established that plant stress and ABA signaling are regulated by proteins that facilitate various processes in RNA metabolism, including transcription elongation mediated by RNA polymerase II (Pol II), mRNA maturation and export, chromatin structure modification, and microRNA production (Hugouvieux et al., 2001; Stockinger et al., 2001; Xiong et al., 2001, 2002; Koiwa et al., 2002; Levy et al., 2002; Vlachonasios et al., 2003; Han et al., 2004; Borsani et al., 2005). Analysis of Arabidopsis (*Arabidopsis thaliana*) mutants that are hyperresponsive to osmotic stresses and ABA has identified a family of *CTD phosphatase-like* (*CPL*) genes that negatively regulate stress-responsive gene expression (Koiwa et al., 2002; Xiong et al., 2002). *CPL1* and *CPL3* were so named because the encoded polypeptides (967 and 1,241 amino acids, respectively)

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are homologous to the FCP1 family of fungal and metazoan protein Ser phosphatases, which regulate transcription by dephosphorylating the carboxyl-terminal domain (CTD) of the largest subunit of Pol II (Lin et al., 2002b).

The CTD of Arabidopsis Pol II contains 34 tandemly repeated heptads with a consensus sequence of $Y^1S^2P^3T^4S^5P^6S^7$ (Nawrath et al., 1990). Dynamic changes in the CTD phosphoarray are controlled by CTD kinases and phosphatases that have Ser-2 and Ser-5 target specificity (Palancade and Bensaude, 2003). Remodeling of the CTD phosphorylation array accompanies the transition from transcriptional initiation to elongation and controls the recruitment, activity, and egress of the various mRNA-processing complexes (Ho and Shuman, 1999; Komarnitsky et al., 2000; Licatalosi et al., 2002; Ahn et al., 2004). Developmental programs and environmental stresses trigger CTD phosphorylation remodeling (Bellier et al., 1997; Dubois and Bensaude, 1998; Bonnet et al., 1999), which is presumed to be a focal process in the differential transcriptional regulation of determinant gene expression.

The Arabidopsis CTD phosphatase gene family is predicted to be composed of more than 20 members based on domain architecture identified in family member proteins of other eukaryotes (Koiwa et al., 2002). CPL3 and CPL4 belong to the FCP1 (TFIIF-interacting CTD phosphatase 1) family of phosphatases that are established to be transcriptional regulators in fungi and metazoa (Archambault et al., 1997, 1998; Hausmann and Shuman, 2002; Lin et al., 2002b). Genetic evidence indicated that CPL3 is a negative regulator of ABA signaling (Koiwa et al., 2002). CPL3 and CPL4 have a conserved N-terminal FCP1 homology (FCPH) domain that contains the consensus DXDXT metal-dependent phosphatase motif and C-terminal breast cancer-1 carboxyl-terminus (BRCT) domain (Archambault et al., 1997; Hausmann and Shuman, 2002). CPL3 has phosphatase activity, but functionality of the BRCT domain is not established. CPL4 functionality is inferred only from sequence homology to CPL3 and other FCP1 homologs. Here, we report the functional dissection of CPL3 and CPL4 proteins in vitro and in vivo. CPL3 and CPL4 are ubiquitously expressed and targeted to nuclei. Molecular interaction of CPL3 or CPL4 with the RAP74 subunit of TFIIF required the C-terminal region that contains the BRCT domain. Loss-of-function/reduced-function genetic analyses indicated that the

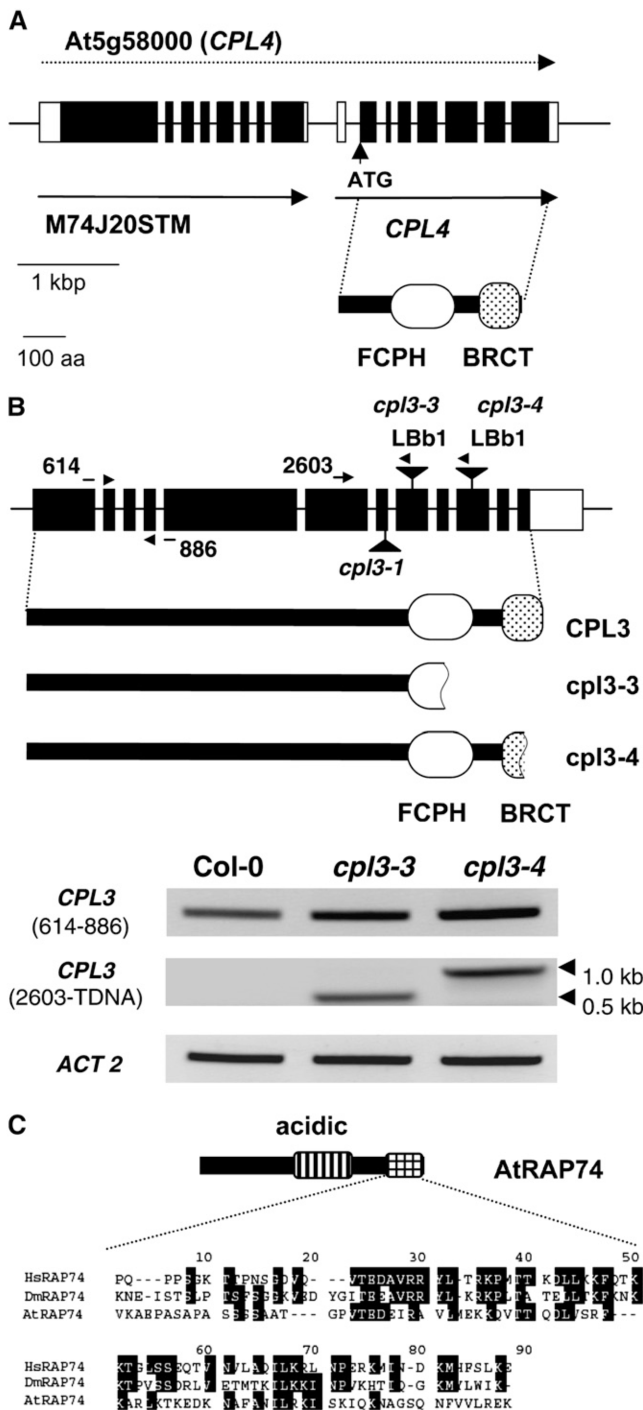

Figure 1. Structure of CPL and RAP74 genes and proteins. A, Schematic drawing of CPL4 (At5g58000) locus. A 2,186-bp 3′ region of annotated At5g58000 encodes *CPL4*. A separate, upstream ORF is confirmed by sequencing EST clone M74JSTM. White and black boxes indicate exons encoding untranslated regions and protein-coding regions. The position of the transcription start site for *CPL4* was determined by 5′ RACE and the initiation codon is indicated by an arrowhead. Predicted CPL4 protein is a 440-amino acid peptide containing FCPH and BRCT domains. B, Structure of new *cpl3* alleles identified in the T-DNA insertion population. T-DNA insertion alleles *cpl3-3* and *cpl3-4* encode proteins truncated at the FCPH and BRCT domains, respectively (top). RT-PCR analysis of *CPL3* transcripts (bottom). Transcripts corresponding to base position 614 to 886 of CPL3 ORF were detected in all the genotypes. *cpl3-3* and *cpl3-4* alleles, but not Columbia-0 (Col-0) wild-type, produce CPL3:T-DNA chimeric transcripts that were detected by CPL3_2603 and LBb1 primers. C, Arabidopsis AtRAP74 is a homolog of the eukaryotic TFIIF large subunit. Schematic drawing of AtRAP74 (top). A striped box and a hatched box indicate the conserved acidic region and C-terminal region, respectively. Alignment of conserved C-terminal regions of RAP74 from Arabidopsis (AAR28013), human (NP_002087), and Drosophila (S30237; bottom). Identical residues are shaded.

BRCT domain is necessary for in vivo function of CPL3 and that CPL4, but not CPL3, is essential for general plant growth. These results establish that these isoforms of a core transcriptional complex have distinct biological functions in plants.

RESULTS

FCP1-Like CTD Phosphatase Complex Components in Arabidopsis

The Arabidopsis genome contains two predicted open reading frames (ORFs), At2g33540 (*CPL3*) and At5g58000 (*CPL4*), which encode proteins with FCP1 domains. We determined previously that *CPL3* encodes a functional phosphatase that regulates stress-responsive transcription and, to a lesser extent, plant growth and development (Koiwa et al., 2002). Genome annotation (The Arabidopsis Information Resource [TAIR]) indicates that At5g58000 encodes CPL4; however, sequence analysis of expressed sequence tag (EST) clone M74J20STM established that polyadenylation occurs at position 2,647 of At5g58000.1 (Fig. 1A), upstream of the predicted phosphatase-coding sequence. The presence of several EST sequences corresponding to the phosphatase region (GenBank accession nos. BX831510, BX832589, BX832758, BX830307, and BX831690) indicates that CPL4 is transcribed from the second ORF separated from the upstream ORF encoding M74J20STM.

Analysis of 5′-RACE products identified the *CPL4* transcription start site at base 23,497,005 of chromosome V. The *CPL4* ORF encodes a 440-amino acid polypeptide that contains a catalytic FCPH domain and a BRCT domain (Fig. 1A). Sequence identities between CPL3 and CPL4 are 44.2% and 54.3% in the FCPH and BRCT domains, respectively. Rice (*Oryza sativa*) genes that encode homologs of CPL3 and CPL4 were identified also (Supplemental Fig. S1). OsCPL3 is 664 amino acids in length and lacks the long N terminus of AtCPL3. However, the overlapping sequences are 52.8% identical. Two rice genes encode CPL4. OsCPL4a and OsCPL4b are 536 and 420 amino acid residues, respectively, and have 41.4% and 37.9% amino acid identity to AtCPL4 polypeptide, respectively (Supplemental Fig. S1).

The RAP74 subunit of the general transcription factor TFIIF interacts and activates FCP1 family phosphatases (Archambault et al., 1997, 1998). A single Arabidopsis gene (At4g12610) encodes a human RAP74 homolog based on BLAST analysis. A cDNA clone encoding AtRAP74 was obtained by reverse transcription (RT)-PCR and the nucleotide sequence was identical to the sequence annotation NM_117331 in GenBank. The ORF of *AtRAP74* encodes a 543-amino acid peptide (Fig. 1C; Supplemental Fig. S2). Two rice homologs, OsRAP74a and OsRAP74b, were identified (Supplemental Fig. S2). The AtRAP74 N terminus is not highly homologous to the corresponding regions in metazoan and fungal RAP74 orthologs. However, the central region (amino acids 231–370) of AtRAP74 is rich in acidic residues (65%), which is a common feature in other RAP74 proteins (Fig. 1C). The C-terminal 108 amino acids of AtRAP74 (position 466–543) has 22% sequence identity to human and Drosophila RAP74 C-terminal regions that interact with cognate FCP1 homologs (Archambault et al., 1997).

CPL3, *CPL4*, and *AtRAP74* were constitutively expressed in both shoots and roots (Fig. 2). *CPL3* only was induced moderately by NaCl treatment, which may be linked to its role in stress-responsive signaling. CPL3, CPL4, and AtRAP74 green fluorescent protein (GFP) fusion proteins were localized exclusively to the nucleus (Fig. 3). The GFP signal was detected within 16 h and nuclear localization did not change throughout the duration of the experiment (3 d).

CPL4 Facilitates Arabidopsis Growth and Development

Only a T-DNA insertion in the *CPL4* 5′-untranslated region (*cpl4-1*: Salk_132900) could be identified and plants lacked any visible phenotypic abnormalities (data not shown). Therefore, RNAi suppression was used to generate *CPL4* reduced expression lines. Fifty-five hygromycin-resistant plants were recovered from two independent transformation experiments and these exhibited a range of phenotypic abnormalities. Germination and seedling establishment comparisons were used to categorize the lines into two classes. Eight independent lines (class I) were normal, whereas the rest (class II) exhibited incomplete to no cotyledon expansion (Fig. 4A, b, c, and i–k). Class II plants grew very slowly and the initial true leaves remained small, had short petioles, and curled downward after the subsequent growth (Fig. 4A, d–g). A group of class II plants (17 lines) subsequently produced leaves that had longer petioles (Fig. 4A, d and e), and developed similarly to wild type. Twenty lines from class II plants matured without recovery from the growth defects and produced small inflorescences (Fig. 4, A and B,

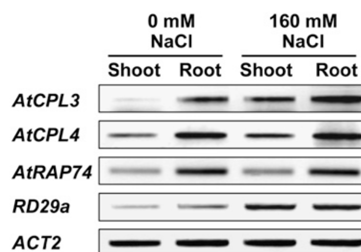

Figure 2. Constitutive and inducible expression of *CPL3*, *CPL4*, and *RAP74*. One-week-old Arabidopsis plants were transferred onto medium with or without 160 mM NaCl and kept for 4 d prior to total RNA extraction. Transcript level for *CPL3*, *CPL4*, *RAP74*, *RD29a*, and *ACT2* genes in shoots and roots were determined by RT-PCR, using 10 μg of total RNA for the RT reaction.

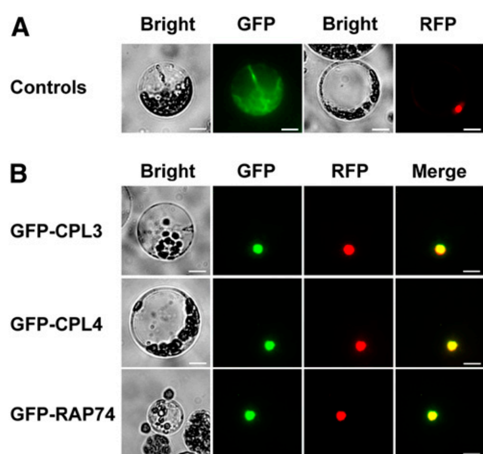

Figure 3. CPL3, CPL4, and RAP74 localize in the nucleus of Arabidopsis. A, Ten micrograms of GFP and red fluorescent protein (RFP)-NLS plasmid were introduced into Arabidopsis protoplasts using the polyethylene glycol method. Three days after transformation, cytoplasmic GFP signal (left) and nuclear RFP signal (right) were observed under a fluorescent microscope. B, Ten micrograms of GFP-CPL3, GFP-CPL4, or GFP-RAP74 plasmid were transformed with RFP-NLS into Arabidopsis protoplasts and their subcellular localization was determined as in A. Bars = 10 μm.

f–h). Ten class II lines produced only three to four true leaves in 30 d and did not reach maturity (Fig. 4A, i–k). Hygromycin-resistant vector control lines (25 lines) did not exhibit any of these anomalies (Fig. 4, A and B, a). Cosegregation of the growth phenotype and hygromycin resistance in viable class II RNAi (*CPL4*) lines was confirmed at the T_2 generation (Table I). All T_2 plants with class II phenotype exhibited hygromycin resistance, whereas all plants with wild-type phenotypes were hygromycin sensitive. These results established that class II growth and developmental phenotypes are linked to the RNAi (*CPL4*) transgene and make it likely that severe growth defects of some class II plants are due to greater suppression of *CPL4* expression in these lines.

The BRCT Domain Is Essential for CPL3 in Vivo Function

CPL3 and CPL4 are distinct CPL isoforms by the presence of the BRCT domain in the C terminus, which is an essential module of metazoan and fungal FCP1 orthologs and is required for catalytic activity of *Schizosaccharomyces pombe* Fcp1. This contrasts with the human small CTD phosphatase 1 (SCP1) family and Arabidopsis CPL1 and CPL2 that require only the FCPH domain for CTD phosphatase activity. To assess the in planta function of the BRCT domain in CPL3, the capacity of *cpl3-3* or *cpl3-4* to suppress the ABA hyperinduction of *RD29a-luciferase* (*LUC*) expression caused by *cpl3-1* (Koiwa et al., 2002) was assessed. The *cpl3-1* T-DNA insertion disrupts the CPL3 catalytic domain. The T-DNA insertion in *cpl3-3* is at position 3,808 from the ATG initiation codon of genomic sequence At2g33540 and the insertion in *cpl3-4* is at position 4,345. *cpl3-3* and *cpl3-4* alleles resulted in the production of chimeric transcripts that could produce proteins that are truncated in the FCPH and BRCT domains, respectively (Fig. 1B). ABA-inducible expression of *RD29a-LUC* in F_1 plants was determined by CCD image analysis (Koiwa et al., 2002). As shown in Figure 5, both alleles failed to complement the ABA hyperresponsiveness caused by *cpl3-1* (i.e. all alleles are equally dysfunctional). Apparently, the 33-amino acid distal region of the BRCT domain is essential for full biological functionality of CPL3. The CPL3 BRCT domain does not include an evident nuclear localization sequence (NLS) as in the yeast (*Saccharomyces cerevisiae*) FCP1 (Kops et al., 2002); consequently, this domain in the plant protein apparently does not function to facilitate nuclear localization.

BRCT Domains of CPL3 and CPL4 Physically Interact with the RAP74 C Terminus

Yeast and human FCP1 contain a central acidic-hydrophobic region and the BRCT domain distal to the

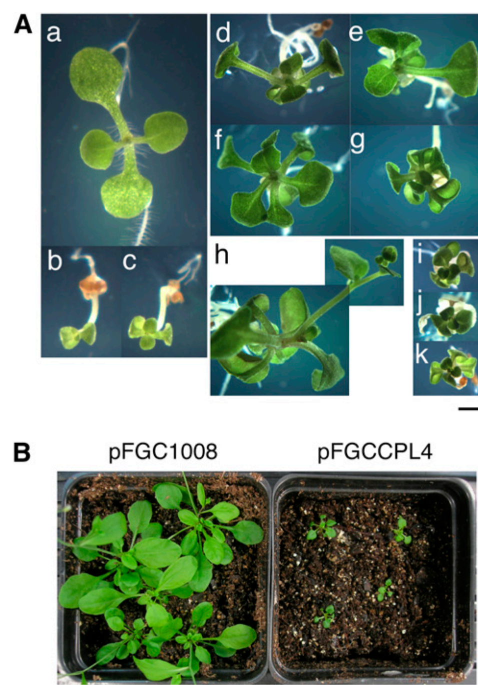

Figure 4. Silencing of *CPL4* by RNAi caused severe growth inhibition of transformants. A, Photograph of 1-week-old (a to c) and 1-month-old (d to k) transgenic plants transformed with a vector (pFGC1008, a) or an RNAi (*CPL4*) construct (pFGCCPL4, b to k) grown in vitro. RNAi (*CPL4*) plants (class II) failed to expand cotyledons after germination (b and c). d and e, Class II plants that start to show elongation of leaf petioles. f to h, Class II plants that have small curled leaves with short petioles. i to k, Class II plants that show senesced cotyledons and unexpanded true leaves. B, Photograph of 1-month-old, flowering-stage transgenic plants grown on soil. Bars = 1 mm (A) and 10 mm (B), respectively.

Table I. *Segregation analysis of RNAi (CPL4) plants in T_2 generation*

Approximately 100 T_2 seeds/line were germinated on 1/4 × Murashige and Skoog medium and scored for wild-type/class II phenotype. The seedlings were then transferred to 1/4 × Murashige and Skoog medium containing 20 μg/mL hygromycin and scored for the hygromycin-resistant (HygR)/sensitive (HygS) phenotype.

Lines	Phenotype			
	Wild Type		Class II	
	HygR	HygS	HygR	HygS
1	0	26	66	0
2	0	30	70	0
3	0	26	74	0
4	0	25	75	0
5	0	24	74	0
6	0	28	70	0

FCPH domain, and this peptide region overlaps with the RAP74-binding region (Archambault et al., 1998). CPL3 and CPL4 catalytic FCPH domains are located in close proximity to the C terminus and the BRCT domain in each protein can be identified on the distal side (Fig. 1). To determine whether the BRCT-containing C-terminal region of CPL3 or CPL4 mediates interaction between CPL and AtRAP74, an immobilized AtRAP74 C-terminal region (77 amino acids) and radiolabeled CPL3 and CPL4 peptide fragments were used to assess in vitro binding. The C-terminal BRCT domain of either CPL3 (amino acids 1,110–1,241) or CPL4 (amino acids 297–440) specifically interacted with immobilized glutathione *S*-transferase (GST)-$RAP74_{466\text{-}543}$ (Fig. 6). A strong interaction is indicated because a significant portion of the labeled CPL peptides remained associated with RAP74 after elution with 0.2 M NaCl. These results establish that the C-terminal BRCT-containing region of CPL3 and CPL4 can associate with the AtRAP74 C-terminal region, which may mediate interaction between CPL and Pol II.

DISCUSSION

The Arabidopsis genome encodes a paralogous pair of *FCP1* homologs, *CPL3* and *CPL4*, unlike those of fungi and metazoans that have only one. Genetic and biochemical results presented herein establish and differentiate the functions of CPL3 and CPL4. Both proteins localize to the nucleus and interact with RAP74 through a peptide region that includes the BRCT domain. Because RAP74 is a TFIIF subunit that is necessary for the interaction between Pol II and FCP1 (Archambault et al., 1997, 1998), these results indicate CPL3 and CPL4 have functions that are similar to other FCP1 proteins. However, gene suppression experiments revealed that *CPL3* and *CPL4* have distinct biological functions. *cpl3* alleles that disrupt either a catalytic FCPH domain or a BRCT domain cause ABA and NaCl hyperresponsive gene expression, but have moderate effects on plant growth and development. RNAi suppression of *CPL4* expression results in growth defects that range from transient retardation of seedling development to lethality prior to maturation. Presumably, the severity of the growth defect is caused by the level of reduced gene expression. This notion is supported indirectly by the fact that T-DNA insertional gene knockout mutations were not identified in any publicly available population. Therefore, it appears that *CPL4* may be essential despite sharing overlapping functions with *CPL3*. This contrasts with another paralogous pair, *CPL1* and *CPL2*, which shares redundant essential functions. Having paralogous FCP1 homologs like CPL3 and CPL4 appears to be ubiquitous in plants because homologs for both CPL3 and CPL4 are present in the rice genome sequence and in EST sequences from various plants, including cotton (*Gossypium hirsutum*), tobacco (*Nicotiana tabacum*), and grape (*Vitis shuttleworthii*). Of these, CPL4 appears to be orthologous to known FCP1 based on the domain structure and its essential function. CPL3 homologs,

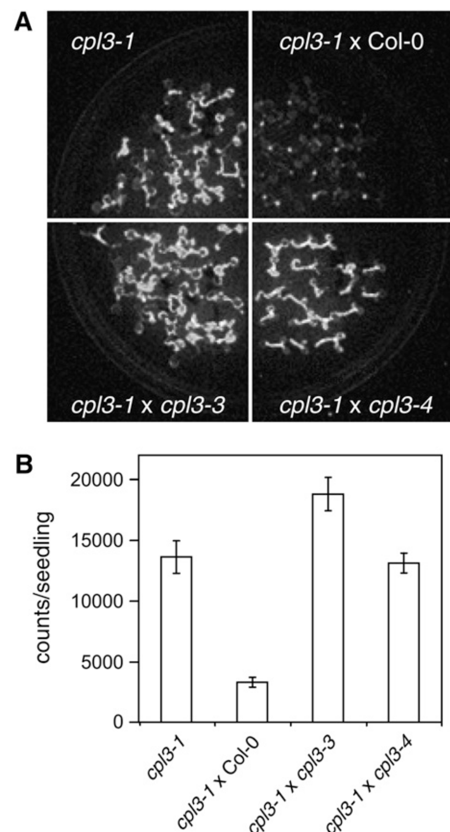

Figure 5. CCD imaging analysis of *RD29a-LUC* reporter gene expression in various *cpl3* mutant alleles. The stigmas from Col-0 *cpl3-3* and Col-0 *cpl3-4* were pollinated with original $C24_{RD29a\text{-}LUC}$ *cpl3-1* plants. F_1 plants were grown for 10 d on 1 × Murashige and Skoog, 3% Suc, and 0.7% agar media. LUC image was taken 3 h after spraying 100 μM ABA (A) and relative luminescence intensity from each genotype was quantified using WinView software (B).

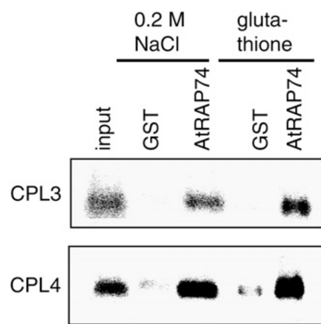

Figure 6. Interaction of BRCT domains from CPL3 and CPL4 with RAP74 C-terminal region. The recombinant GST-RAP74$_{466\text{-}543}$ and GST were immobilized on glutathione sepharose and were incubated with ACB buffer containing in vitro synthesized, radiolabeled BRCT domain peptides of CPL3 or CPL4. The unbound peptides were washed with ACB buffer. Bound peptides were sequentially eluted by 0.2 M NaCl and reduced glutathione. One-hundredth input solution (input) and bound peptides in each elution were resolved by tricine SDS-PAGE, and detected by phosphor imager.

on the other hand, may function in transducing specific environmental and developmental signals, such as ABA response. Consistent with this hypothesis, a *CPL3* homolog of tomato (*Lycopersicon esculentum*) is a candidate gene for a *jointless2* locus that has very specific developmental function, namely, the formation of an abscission zone in fruits (Yang et al., 2005).

CPL3 is the largest known FCP1-like protein (1,242 amino acids), which is attributable to a long N-terminal region preceding the FCPH domain. Located in the CPL3 N terminus is a short region that exhibits homology to yeast Ces1, a multicopy suppressor of *cgl1* mutations of the mRNA-capping enzyme gene (Scwer et al., 1998; Koiwa et al., 2002). CPL4 is only 29 amino acids longer than the smallest FCP1 ortholog, which was identified in the microsporidian parasite *Encephalitozoon cuniculi* (Hausmann et al., 2004). This structural diversity may be indicative of different kinetic properties or protein-protein interaction specificity. CPL3 and CPL4 belong to the FCP1 family that requires Pol II holoenzyme and RAP74 for its CTD phosphatase activity (Archambault et al., 1997, 1998; Kobor et al., 1999). Because strict substrate specificity exists between FCP1 homologs and Pol II phospho-CTD (Lin et al., 2002a; Yeo et al., 2003), further biochemical characterization of CPL3 and CPL4 may require the use of a holocomplex involving at least intact Pol II and AtRAP74.

How do CPL3 and CPL4 (and other CPLs [Yang et al., 2005]) differentially regulate the transcriptome? Recent studies on metazoans indicate that CTD phosphatases interact with specific transcription factors, a process by which the phosphatase is recruited to target gene promoters to regulate expression. For example, Drosophila and human SCPs are recruited specifically by the zinc-finger transcriptional repressor REST/NRSF complex to silence neuronal genes in nonneuronal cells (Yeo et al., 2005). The CPL enzyme, Drosophila eyes absent (Eya), interacts with a homeodomain transcription factor sine oculis and activates expression of genes that are necessary for eye formation. A CTD phosphatase that is recruited to a specific promoter region in this

Table II. *Sequences of primers used in this study*

Primer	Sequence
CPL3F	gaagatctatgcttgtagctcgatctggt
CPL3R	aggaagcggccgcttacgggttgatggcatataagttct
CPL3RTF	ttgctagccttctccaaaaa
CPL3RTR	gaggatgtgtcagtttcagcagc
CPL3_614	aagcacagaccggatttg
CPL3_886	tggtctctatctcatttttctgg
CPL3_2603	gaggatgtgtcagtttcagcagc
CPL4F	gaagatctatgagcgtagcaagtgattctcca
CPL4R	aggaagcggccgcgctatctcttctttctcattgttgttac
CPL4N8	accgtcgtgacatctccactc
CPL4RTF	ctattctttgagaatgtgga
CPL4RTR	ttagatcttcactcttcttcggtcaattgct
CPL4RTMA1	tgcttgaggatgcttcctctaaat
CPL4RTMA2	gagcctcatttccttgtgtatg
LBb1	gcgtggaccgcttgctgcaact
RAP74F	gaggatccatgtcgaactgtttgcaattgaatacgtc
RAP74R	aggagtcgaccttttctctcaaaacgacgaagttttgag
RAP74CF1	agctgaaccagcttcagctcctgcat
RAP74CR1	tcacttttctctcaaaacgacgaagttttgagaaccagcgt
RAP74CF2	gtcagctgcgactgggcctgtgactga
RAP74CR2	tcacttttctctcaaaacgacgaagttttgagaacc
T7-MTAG	ctagagtacttaatacgactcactataggcgaatacaagctacttgttctttttgcaccctcatggaaatgatgacaatg
T7-TERM	caaaaaacccctcaagacccgtttagaggccccaaggggttatgctagttattgctcagcggt
M-CPL3	atggaaatgatgacaatgtcctctatgatggaggagggcacattg
T7-CPL3	atgctagttattgctcagcggtttacgggttgatggcatat
M-CPL4	atggaaatgatgacaatgtcctctatgatgagtgaaccagatggg
T7-CPL4	atgctagttattgctcagcggttcactcttcttcggtcaattg

manner may regulate transcription by dephosphorylating Pol II CTD that is phosphorylated by TFIIH (Yeo et al., 2003) or the transcription factor associated with specific target genes (Li et al., 2003; Tootle et al., 2003). By analogy, CPL3 that specifically regulates ABA signaling may be recruited to ABA-responsive promoters by transcription factors and fine-tuned transcription. Potential partners/substrates of CPL3 besides Pol II CTD include zinc-finger transcription factors ABF/AREB that are regulated by phosphorylation/dephosphorylation during ABA-responsive transcription or the homeodomain transcription factor HOS9 and MYB-domain transcription factor HOS10, which were identified in the same mutational screen as *cpl3-1* (Zhu et al., 2004, 2005).

Interaction between CPL3/CPL4 and AtRAP74 indicates that both isoforms likely associate with the elongating Pol II complex. Differential regulation of gene expression by CPL isoforms, therefore, may occur also at the level of mRNA elongation and processing. Fine tuning of the CTD phosphoarray in the elongation complex may affect recruitment of RNA-processing factors and production of mature transcripts. Splicing factors such as STABILIZED (Lee et al., 2006) and FCA (Razem et al., 2006) that are involved in osmotic stress or ABA signaling potentially may be some of the recruited regulators. Indeed, FCA interacts with FY, an Arabidopsis homolog of a CTD-binding protein CstF50 (Simpson et al., 2003; Razem et al., 2006). The presence of an ABA-specific CTD phosphatase CPL3 strongly supports the notion that a specific CTD phosphoarray is required to define the signature of the transcriptional output during ABA response.

MATERIALS AND METHODS

Materials

Arabidopsis (*Arabidopsis thaliana*) plants for protoplast preparation were grown on agar plates containing 1/3× Murashige and Skoog salts and 0.5% Suc. The salt treatment of Arabidopsis seedlings was conducted as described (Koiwa et al., 2003). The pENSOTG GFP fusion vector has been prepared previously (Koiwa et al., 2004). pUCNLSDsRed was provided by Dr. I. Hwang (Jin et al., 2001). Primer sequences used in this research are listed in Table II.

cDNA Cloning and Preparation of GFP Expression Constructs

The transcription start site for CPL4 was determined by 5′-RACE analysis using the 5′-RACE full core set (TaKaRa). Full-length ORF sequences of CPL3, CPL4, and AtRAP74 cDNAs were amplified by PCR (for CPL3) or RT-PCR with primer sets CPL3F and CPL3R, CPL4F and CPL4R, and RAP74F and RAP74R, respectively, then cloned in pET44a between *Bam*HI-*Not*I sites. For protoplast transformation, the *Sma*I-*Not*I fragment of CPL3, CPL4, and RAP74 was excised from pET44a-CPL and pETRAP74 plasmids and inserted in pENSOTG, resulting in pENCPL3, pENCPL4, and pENRAP74.

Expression Analysis by RT-PCR

Total RNA was isolated from Arabidopsis seedlings using the RNeasy plant mini kit (Qiagen). RT-PCR was performed as described previously (Koiwa et al., 2003) using primer sets CPL3RTF and CPL3RTR, CPL4RTF and CPL4RTR, and RAP74CF2 and RAP74CR2. RD29a and ACT2 primers were used as positive controls (Koiwa et al., 2003). For analysis of truncated CPL3 transcripts in *cpl3-3* and *cpl3-4*, RT-PCR was performed using primer pairs CPL3_614 and CPL3_886 for the intact 5′ region and CPL3_2603 and LBb1 for chimeric transcripts.

Protoplast Purification and Transformation

Isolation and transformation of Arabidopsis protoplasts with pENCPL3, pENCPL4, and pENRAP74 plasmids were conducted as described (Koiwa et al., 2004). Transformed protoplasts were incubated at 22°C in the dark. Expression of the fusion constructs was evaluated at 2 and 3 d after transformation as described (Koiwa et al., 2004).

RNAi Analysis

The RNAi construct was prepared by inserting the CPL4 cDNA fragment (position 315–849 bp) into pFGC1008 (http://www.chromdb.org). Resulting pFGCCPL4 was transformed into *Agrobacterium tumefaciens* GV3101 and was used for flower transformation of Arabidopsis. Transgenic plants were selected on media containing 1/4× Murashige and Skoog salts and 20 μg/mL hygromycin B and 100 μg/mL cefotaxim.

Cosegregation of the transgene and growth phenotype was performed using T_2 progeny of viable RNAi (*CPL4*) lines. Six independent class II lines that contain single-copy T-DNA were used for the analysis. Approximately 100 seeds were grown on media containing 1/4× Murashige and Skoog salts and 0.8% agar for 1 week. The growth phenotype was scored for each plant and then the entire population was transferred to the same media containing 20 μg/mL hygromycin B. Resistance to hygromycin B was scored 5 d after the transfer.

Genetic Analysis

cpl3-3 and *cpl3-4* stigmas were pollinated with *cpl3-1*pollens that contain the *RD29a-LUC* reporter gene along with a mutated *CPL3* allele. ABA-inducible expression of the LUC reporter gene was analyzed in F_1 plants as described (Koiwa et al., 2002).

Pull-Down Assay

A cDNA fragment encoding an AtRAP74 C-terminal fragment, $RAP74_{466-543}$, was amplified by RT-PCR with primers RAP74CF1 and RAP74CR1 and was inserted into the *Sma*I site of pGEX-2T (GE Healthcare). Expression of GST fusion proteins or control GST protein in *Escherichia coli* was induced by 0.4 mM isopropylthio-β-galactoside at 37°C for 4 h, and cells were harvested by centrifugation. Cells were resuspended in Tris-buffered saline (10 mM Tris, 0.9% NaCl) and disrupted by sonication. Crude lysate containing approximately 40 μg GST fusion protein was loaded onto a microspin GST column (Amersham) and unbound protein was removed by washing the beads in Tris-buffered saline. The beads were blocked with 3% bovine serum albumin in ACB buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM DTT, 10% glycerol) at 4°C for 1 h and washed with ACB wash buffer (ACB buffer containing 0.1 M NaCl; Archambault et al., 1997).

Radiolabeled CPL peptides were prepared using the coupled in vitro transcription/translation system (Promega). cDNA fragments corresponding to the BRCT domain of CPL3 and CPL4 were amplified by two rounds of PCR (first round: M-CPL3, T7-CPL3; M-CPL4, T7-CPL4; second round: M-TAG, T7-TERM). The resulting PCR products contain a T7 promoter sequence and initiation codon followed by an M-tag (MEMMTMSSMM) at the 5′ end, and a T7 terminator sequence at the 3′ end of CPL BRCT domains. The M-tag sequence was used to increase the labeling efficiency by [S^{35}]-Met. PCR products were purified from agarose gel and used as templates for in vitro transcription/translation according to the manufacturer's protocol.

Ten microliters of in vitro translation products were diluted with 40 μL of ACB buffer and added to the glutathione-agarose beads loaded with GST-RAP74 or GST by itself at 4°C for 30 min. After washing with ACB wash buffer (Archambault et al., 1997), bound peptides were eluted by 100 μL 0.2 M NaCl and then 10 mM reduced glutathione, resolved by 12% tricine SDS-PAGE (Schägger and von Jagow, 1987), and were detected by phosphor imager (Molecular Dynamics).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF486633 (AtCPL3), DQ503426 (AtCPL4),

ABA93957 (OsCPL3), AAS86390 (OsCPL4a), XP_468260 (OsCPL4b), NM117331 (AtRAP74), AAX95774 (OsRAP74a), AK073337 (OsRAP74b), NP_002087 (human RAP74), and S30237 (Drosophila RAP74).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Amino acid sequence comparison of Arabidopsis and rice CPL3 and CPL4 homologs.

Supplemental Figure S2. Amino acid sequence comparison of Arabidopsis and rice RAP74 homologs.

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LITERATURE CITED

Ahn SH, Kim M, Buratowski S (2004) Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3′ end processing. Mol Cell **13:** 67–76

Archambault J, Chambers R, Kobor MS, Ho Y, Bolotin D, Andrews B, Kane CM, Greenblatt J (1997) An essential component of a C-terminal domain phosphatase that interacts with transcription factor IIF in *Saccharomyces cerevisiae.* Proc Natl Acad Sci USA **94:** 14300–14305

Archambault J, Pan G, Dahmus GK, Cartier M, Marshall N, Zhang S, Dahmus ME, Greenblatt J (1998) FCP1, the RAP74-interacting subunit of a human protein phosphatase that dephosphorylates the carboxyl-terminal domain of RNA polymerase II. J Biol Chem **273:** 27593–27601

Bellier S, Dubois MF, Nishida E, Almouzni G, Bensaude O (1997) Phosphorylation of the RNA polymerase II largest subunit during Xenopus laevis oocyte maturation. Mol Cell Biol **17:** 1434–1440

Bonnet F, Vigneron M, Bensaude O, Dubois MF (1999) Transcription-independent phosphorylation of the RNA polymerase II C-terminal domain (CTD) involves ERK kinases (MEK1/2). Nucleic Acids Res **27:** 4399–4404

Borsani O, Zhu J, Verslues PE, Sunkar R, Zhu JK (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. Cell **123:** 1279–1291

Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, Agarwal M, Zhu JK (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. Genes Dev **17:** 1043–1054

Choi H, Hong J, Ha J, Kang J, Kim SY (1999) ABFs, a family of ABA-responsive element binding factors. J Biol Chem **275:** 1723–1730

Dubois MF, Bensaude O (1998) Phosphorylation of RNA polymerase II C-terminal domain (CTD): a new control for heat shock gene expression? Cell Stress Chaperones **3:** 147–151

Fowler S, Thomashow MF (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cp;d response pathway. Plant Cell **14:** 1675–1690

Han MH, Goud S, Song L, Fedoroff N (2004) The Arabidopsis double-stranded RNA-binding protein HYL1 plays a role in microRNA-mediated gene regulation. Proc Natl Acad Sci USA **101:** 1093–1098

Hasegawa PM, Bressan RA, Zhu J-K, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol **51:** 463–499

Hausmann S, Schwer B, Shuman S (2004) An Encephalitozoon cuniculi ortholog of the CTD serine phosphatase Fcp1. Biochemistry **43:** 7111–7120

Hausmann S, Shuman S (2002) Characterization of the CTD phosphatase Fcp1 from fission yeast: preferential dephosphorylation of serine 2 versus serine 5. J Biol Chem **277:** 21213–21220

Ho CK, Shuman S (1999) Distinct roles for CTD Ser-2 and Ser-5 phosphorylation in the recruitment and allosteric activation of mammalian mRNA capping enzyme. Mol Cell **3:** 405–411

Hugouvieux V, Kwak JM, Schroeder JI (2001) An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in *Arabidopsis.* Cell **106:** 477–487

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. Science **280:** 104–106

Jin JB, Kim YA, Kim SJ, Lee SH, Kim DH, Cheong GW, Hwang I (2001) A new dynamin-like protein, ADL6, is involved in trafficking from the trans-Golgi network to the central vacuole in Arabidopsis. Plant Cell **13:** 1511–1526

Kang JY, Choi HI, Im MY, Kim SY (2002) Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell **14:** 343–357

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol **17:** 287–291

Kobor MS, Archambault J, Lester W, Holstege FCP, Gileadi O, Jansma DB, Jennings EG, Kouyoumdjian F, Davidson AR, Young RA, et al (1999) An unusual eukaryotic protein phosphatase required for transcription by RNA polymerase II and CTD dephosphorylation in *S. cerevisiae.* Mol Cell **4:** 55–62

Koiwa H, Barb AW, Xiong L, Li F, McCully MG, Lee B-h, Sokolchik I, Zhu J, Gong Z, Reddy M, et al (2002) C-terminal domain phosphatase-like family members (AtCPLs) differentially regulate *Arabidopsis thaliana* abiotic stress signaling, growth, and development. Proc Natl Acad Sci USA **99:** 10893–10898

Koiwa H, Hausmann S, Bang WY, Ueda A, Kondo N, Hiraguri A, Fukuhara T, Bahk JD, Yun DJ, Bressan RA, et al (2004) Arabidopsis C-terminal domain phosphatase-like 1 and 2 are essential Ser-5-specific C-terminal domain phosphatases. Proc Natl Acad Sci USA **101:** 14539–14544

Koiwa H, Li F, McCully MG, Mendoza I, Koizumi N, Manabe Y, Nakagawa Y, Zhu J-H, Rus A, Pardo JM, et al (2003) The STT3a subunit isoform of the *Arabidopsis thaliana* oligosaccharyltransferase controls adaptive responses to salt/osmotic stress. Plant Cell **15:** 2273–2284

Komarnitsky P, Cho EJ, Buratowski S (2000) Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. Genes Dev **14:** 2452–2460

Kops O, Zhou XZ, Lu KP (2002) Pin1 modulates the dephosphorylation of the RNA polymerase II C-terminal domain by yeast Fcp1. FEBS Lett **513:** 305–311

Lee BH, Kapoor A, Zhu J, Zhu JK (2006) STABILIZED1, a stress-up-regulated nuclear protein, is required for pre-mRNA splicing, mRNA turnover, and stress tolerance in Arabidopsis. Plant Cell **18:** 1736–1749

Levy YY, Mesnage S, Mylne JS, Gendall AR, Dean C (2002) Multiple roles of Arabidopsis VRN1 in vernalization and flowering time control. Science **297:** 243–246

Li X, Oghi KA, Zhang J, Krones A, Bush KT, Glass CK, Nigam SK, Aggarwal AK, Maas R, Rose DW, et al (2003) Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. Nature **426:** 247–254

Licatalosi DD, Geiger G, Minet M, Schroeder S, Cilli K, McNeil JB, Bentley DL (2002) Functional interaction of yeast pre-mRNA 3′ end processing factors with RNA polymerase II. Mol Cell **9:** 1101–1111

Lin PS, Dubois MF, Dahmus ME (2002a) TFIIF-associating carboxyl-terminal domain phosphatase dephosphorylates phosphoserines 2 and 5 of RNA polymerase II. J Biol Chem **277:** 45949–45956

Lin PS, Marshall NF, Dahmus ME (2002b) CTD phosphatase: role in RNA polymerase II cycling and the regulation of transcript elongation. Prog Nucleic Acid Res Mol Biol **72:** 333–365

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought and low-temperature gene expression, respectively, in Arabidopsis. Plant Cell **10:** 1391–1406

Maruyama K, Sakuma Y, Kasuga M, Ito Y, Seki M, Goda H, Shimada Y, Yoshida S, Shinozaki K, Yamaguchi-Shinozaki K (2004) Identification of cold-inducible downstream genes of the Arabidopsis DREB1A/CBF3 transcriptional factor using two microarray systems. Plant J **38:** 982–993

Nawrath C, Schell J, Koncz C (1990) Homologous domains of the largest subunit of eucaryotic RNA polymerase II are conserved in plants. Mol Gen Genet **223:** 65–75

Palancade B, Bensaude O (2003) Investigating RNA polymerase II carboxyl-terminal domain (CTD) phosphorylation. Eur J Biochem **270:** 3859–3870

Razem FA, El-Kereamy A, Abrams SR, Hill RD (2006) The RNA-binding protein FCA is an abscisic acid receptor. Nature **439:** 290–294

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem **166:** 368–379

Scwer B, Linder P, Shuman S (1998) Effects of deletion mutations in the yeast Ces1 protein on cell growth and morphology and on high copy suppression of mutations in mRNA capping enzyme and translation initiation factor 4A. Nucleic Acids Res **26:** 803–809

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, et al (2002) Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J **31:** 279–292

Simpson GG, Dijkwel PP, Quesada V, Henderson I, Dean C (2003) FY is an RNA 3′ end-processing factor that interacts with FCA to control the Arabidopsis floral transition. Cell **113:** 777–787

Stockinger E, Gilmour SJ, Thomashow MF (1997) *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription response to low temperature and water deficit. Proc Natl Acad Sci USA **94:** 1035–1040

Stockinger EJ, Mao Y, Regier MK, Triezenberg SJ, Thomashow MF (2001) Transcriptional adaptor and histone acetyltransferase proteins in Arabidopsis and their interactions with CBF1, a transcriptional activator involved in cold-regulated gene expression. Nucleic Acids Res **29:** 1524–1533

Tootle TL, Silver SJ, Davies EL, Newman V, Latek RR, Mills IA, Selengut JD, Parlikar BE, Rebay I (2003) The transcription factor Eyes absent is a protein tyrosine phosphatase. Nature **426:** 299–302

Uno Y, Furihara T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2000) *Arabidopsis* basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proc Natl Acad Sci USA **97:** 11632–11637

Viswanathan C, Zhu JK (2002) Molecular genetic analysis of cold-regulated gene transcription. Philos Trans R Soc Lond B Biol Sci **357:** 877–886

Vlachonasios KE, Thomashow MF, Triezenberg SJ (2003) Disruption mutations of ADA2b and GCN5 transcriptional adaptor genes dramatically affect Arabidopsis growth, development, and gene expression. Plant Cell **15:** 626–638

Xiong L, Gong Z, Rock CD, Subramanian S, Guo Y, Xu W, Galbraith D, Zhu J-K (2001) Modulation of abscisic acid signal transduction and biosynthesis by an Sm-like protein in *Arabidopsis.* Dev Cell **1:** 771–781

Xiong L, Lee H, Ishitani M, Tanaka Y, Stevenson B, Koiwa H, Bressan RA, Hasegawa PM, Zhu J-K (2002) Repression of stress-responsive genes by FIERY2, a novel transcriptional regulator in Arabidopsis. Proc Natl Acad Sci USA **99:** 10899–10904

Yamaguchi-Shinozaki K, Shinozaki K (2005) Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. Trends Plant Sci **10:** 88–94

Yang TJ, Lee S, Chang SB, Yu Y, de Jong H, Wing RA (2005) In-depth sequence analysis of the tomato chromosome 12 centromeric region: identification of a large CAA block and characterization of pericentromere retrotranposons. Chromosoma **114:** 103–117

Yeo M, Lee SK, Lee B, Ruiz EC, Pfaff SL, Gill GN (2005) Small CTD phosphatases function in silencing neuronal gene expression. Science **307:** 596–600

Yeo M, Lin PS, Dahmus ME, Gill GN (2003) A novel RNA polymerase II C-terminal domain phosphatase that preferentially dephosphorylates serine 5. J Biol Chem **278:** 26078–26085

Zhu J, Shi H, Lee BH, Damsz B, Cheng S, Stirm V, Zhu JK, Hasegawa PM, Bressan RA (2004) An Arabidopsis homeodomain transcription factor gene, HOS9, mediates cold tolerance through a CBF-independent pathway. Proc Natl Acad Sci USA **101:** 9873–9878

Zhu J-H, Verslues PE, Zheng X, Lee B-H, Zhan X, Manabe Y, Sokolchik I, Zhu Y, Dong C-H, Zhu J-K, et al (2005) HOS10 encodes a novel R2R3-type MYB transcription factor essential for cold acclimation in plants. Proc Natl Acad Sci USA **102:** 9966–9971