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Regulation of aleurone cell fate determinants in Zea mays

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Regulation of aleurone cell fate determinants in *Zea mays*

by

**Antony Mathai Chettoor**

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

**DOCTOR OF PHILOSOPHY**

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2009

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Dedicated to:

My parents and family
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CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

Human nutrition is provided by a limited number of plant species. About 90% of mankind’s food supply is derived from approximately 17 species, of which cereal grains supply the greatest percentage. Wheat, maize and rice together comprise at least 75% of the world’s grain production (Cordain 1999). The primary nutritious part of the cereal grain is the seed endosperm. Despite detailed knowledge of events that occur in angiosperm fertilization and endosperm formation, very little is known about the regulatory networks controlling the complex developmental and metabolic processes of cereal grain formation.

In cereal plants, double fertilization initiates the process of seed formation, in which one sperm nucleus fertilizes the egg cell in the embryo sac resulting in a diploid zygote, a second sperm nucleus fuses with two polar nuclei of the central cell to initiate the development of the triploid endosperm (Dumas and Mogensen 1993). The diploid zygote and the primary triploid nucleus enter separate developmental patterns to give rise to the embryo and the nutritive endosperm. The pathway leading to the formation of the endosperm from the triploid nucleus is a four stage process. (1) syncytial stage, where the primary triploid nucleus in the central cell undergoes a period of mitotic nuclear divisions without cytokinesis resulting in a large syncytium; (2) cellularization, a period during which cytokinesis separates the nuclei into discrete cells involving both anticlinal and periclinal divisions; (3) growth and differentiation, which results in distinct tissues namely starchy endosperm, basal transfer layer and aleurone and (4) maturation, an important process characterized by
accumulation of storage reserves and the development of desiccation tolerance and dormancy (Becraft 2001, Olsen 2001).

Aleurone Cell Development

Cereal endosperm consists of three major cell types, the starchy endosperm cells, the transfer cells and the aleurone (Becraft 2001, Olsen 2001). The starchy endosperm is the major storage cell type and constitutes the bulk of the endosperm. These cells accumulate massive amounts of starch and protein deposited in starch grains and protein bodies. The aleurone layer is the peripheral cell layer of the endosperm. In most cereals, the aleurone is a single layer of cells, but in barley and some lines of rice it consists of 3-4 cell layers. In maize, the aleurone cells are cuboidal in shape, high in lipids, hydrolytic enzymes and proteins. In Arabidopsis, only one layer of aleurone-like cells is left on the periphery as endosperm degenerates and is reabsorbed into the embryo (Bethke et al. 2007, Olsen 2001). In cereals, the aleurone layer functions primarily as a digestive tissue that secretes amylases and proteases that degrade the starch and protein stored in the underlying starchy endosperm cells. Aleurone cells also serve a storage function; they are a major site for phosphorus storage in the form of phytic acid (Pilu et al. 2003), and are rich in lipids and proteins (Jones 1969). The basal endosperm transfer layer (BETL) forms at the base of the endosperm adjacent to the maternal vasculature to transport solutes from the maternal tissues into the growing endosperm. Transfer cells contain distinct laminar cell wall ingrowths that dramatically increase the plasmamembrane surface area to perform this transport function (Offler et al. 2003, Talbot et al. 2002, Thompson et al. 2001).
Aleurone identity is not fixed and specification of aleurone fate occurs continually through the later stages of development. Mosaic analyses showed that the same parental cells can result in either aleurone or starch cell fate and do not possess unique cell lineages (Becraft and Asuncion-Crabb 2000). This implies that positional cues are continuously required during development to specify and maintain aleurone cell fate in the peripheral layer. In particular maize genotypes, during the differentiation process the aleurone is pigmented due to the accumulation of anthocyanin, a phenolic pigment (Cone et al. 1986, Ludwig and Wessler 1990). Anthocyanin also serves as an excellent genetic marker for studying cell fate specification of aleurone cells. The seeds become colorless when peripheral cells of mutant kernels lose the aleurone cell identity (Becraft 2002, Becraft and Asuncion-Crabb 2000). Based on the collection of aleurone mutants and using anthocyanin as a visual marker, a putative genetic hierarchy that regulates the development of aleurone cells has been proposed (Becraft and Asuncion-Crabb, 2000) (Figure 1). Broadly the mutants can be placed in two categories; cell fate mutants and aleurone differentiation mutants. Cell fate mutants define early factors and aleurone differentiation mutants define factors late in the aleurone development. The model proposes, *defective kernel1* (dek1), *crinkly4* (cr4), and *naked endosperm* (nkd) as cell fate mutants while *Dappled1* (DAP1) and *viviparous-1* (vp1) fall in the category of aleurone differentiation mutants (Becraft 2002, Becraft and Asuncion-Crabb 2000, Gavazzi et al. 1997, Lid et al. 2002). In cell fate mutants like dek1 and cr4, the peripheral aleurone cell layer is lost and acquires a starchy endosperm cell identity. The nkd mutant appears to affect an early step in the aleurone differentiation process. In this mutant, the peripheral endosperm cell layer has characteristics distinct from starchy endosperm, but lack true aleurone characteristics. In mutants like DAP1, later steps in aleurone cell
differentiation are affected. The aleurone has relatively mild morphological defects along with patchy pigmentation. In maize and cereal grains the aleurone undergoes a maturation process late in development during which the aleurone and the embryo acquires dormancy and remains viable as dry seed. Mutations in the \textit{vp1} locus cause loss of dormancy resulting in precocious seed germination and loss of aleurone pigmentation (McCarty \textit{et al.} 1989). \textit{Vp1} encodes a transcription factor and represents the most upstream known transcription factor in the aleurone anthocyanin pathway.

Maize \textit{crinkly4 (Cr4)} encodes a functional serine/threonine receptor-like kinase (RLK) and plays an important role in an array of developmental processes both in the plant and in the aleurone (Becraft and Asuncion-Crabb 2000, Becraft \textit{et al.} 1996, Jin \textit{et al.} 2000). In the plant, CR4 regulates cell proliferation, fate, patterning, morphogenesis, and differentiation, particularly in the leaf epidermis. In the endosperm, CR4 is required for aleurone cell fate specification and the \textit{cr4} mutant results in peripheral aleurone cell layer replaced by starchy endosperm. Associated with the loss of aleurone cell identity, the mutant kernels exhibits a mosaic anthocyanin pigmented aleurone phenotype. The CR4 protein domain organization includes a signal sequence, seven copies of the 39-amino acid “crinkly” repeat, a cysteine rich region similar to the mammalian tumor necrosis factor receptor (TNFR), a transmembrane domain, the catalytic domain of a serine/threonine protein kinase and carboxy-terminal region (Becraft, Stinard and McCarty 1996). The “crinkly” repeat along with the TNFR-like domains represents the extracellular domains. The serine/threonine protein kinase and carboxy-terminal regions represent the cytoplasmic domains.

Maize \textit{Cr4} is represented as a single copy in the maize genome as well as the rice and \textit{Arabidopsis} genomes. In Arabidopsis, a family of five RLKs closely related to maize CR4
has been identified (Cao et al. 2005, Gifford et al. 2003, Tanaka et al. 2002). Arabidopsis CR4 (ACR4) represents the closest ortholog to ZmCR4 with 60% amino acid identity. The ACR4 protein exhibits identical domain organization as that of ZmCR4 including the extracellular “crinkly repeats”, TNFR repeats and cytoplasmic kinase and carboxyl domain. The other four Arabidopsis homologs are designated as AtCRR for Arabidopsis thaliana CR4-RELATED (AtCRR1, AtCRR2, AtCRR3 and AtCRR4). AtCRR4 turned out to be similar to CRK1 in tobacco and was named AtCRK1. All the CRRs contain the “crinkly repeats” but lack the carboxyl domain. AtCRR1 and AtCRR2 contain TNFR repeats, but are predicted to contain inactive kinases because of a conserved deletion that removes essential residues of the kinase subdomain. AtCRR3 and AtCRK1 lack TNFR repeats in the extracellular domains, but contain kinase domains that are predicted to be functional. T-DNA insertions in ACR4 exhibited defects in the development of the integuments and seed coat (Gifford et al. 2003, Tanaka et al. 2002). The acr4 mutant also showed disruption of cell organization in leaf epidermis and cuticle formation (Watanabe et al. 2004). The lack of severe phenotypic defects in acr4 mutants compared to maize cr4 is surprising considering the high sequence conservation between the maize and Arabidopsis CR4 proteins. This could be explained by functional redundancy in Arabidopsis, if the mutants are not completely null or the lesions in these mutants do not affect critical functional subdomains of the protein.

In addition to the unique structural attributes of the CR4 receptor, ACR4 appears to be rapidly degraded when active in signaling. Wild type ACR4 protein is nearly undetectable on immunoblots, whereas nonfunctional protein variants accumulate to readily detectable levels (Gifford et al. 2005). Gifford and co-workers demonstrated that wild type ACR4 protein or variants that complement an acr4 mutant are nearly undetectable on immunoblots,
whereas nonfunctional protein variants accumulate to readily detectable levels. The rapid turnover of functional ACR4 sets it apart from other known plant receptor kinases. An understanding of the regulation and cellular dynamics of the CR4 receptor turnover will help us better understand the signal transduction events that occur in aleurone cell fate specification.

The Function of Vp1 in Aleurone and Seed Maturation

In seed development, the maturation stage marks the switch from maternal controls to that of the growing seed (Weber et al. 2005). This switch coincides with the accumulation of macromolecular reserves, such as storage proteins, lipids, and carbohydrates. Towards the end of this accumulation stage, starchy endosperm undergoes programmed cell death, while both the embryo and the aleurone remain alive, acquire desiccation tolerance, and enter developmental arrest. Mature embryos remain metabolically dormant until favorable conditions trigger germination. Proper control of the maturation transition is critical to the survival of the plant in nature. From an agronomic perspective, the maturation phase is crucial because of its relationship to dormancy and preharvest sprouting.

Plant hormones like Abscisic acid (ABA) and gibberellic acid (GA) are known regulators of the dormancy to germination transition. Abscisic acid (ABA) is well known to promote seed dormancy and repress seed germination, whereas gibberellic acid functions to initiate seed germination (Finch-Savage and Leubner-Metzger 2006). During the maturation phase of seed development, among the many maturation-associated genes induced by ABA, *late embryogenesis abundant* (Lea) genes have been of interest. LEA proteins are ubiquitous in plants. They accumulate in the seed during the late stages of seed development as well as
in vegetative tissues under drought, heat, cold and salt stress conditions or with ABA application. LEA proteins functionally are shown to play a role in the tolerance to desiccation by maintaining the structural integrity of membranes and proteins and controlling water exchange (Dure 1993). In addition to the LEA’s, ABA regulates three plants-specific B3 domain transcription factors: ABSCISIC ACID INSENSITIVE 3 (ABI3), FUSCA3 (FUS3) and LEAFY COTYLEDON 2 (LEC2)(Finch-Savage and Leubner-Metzger 2006, Finkelstein et al. 2002). In the loss- of - function ABI3, FUS3 and LEC2 mutants the embryo maturation program is absent, and the acquisition of desiccation tolerance in the developing seed is lost. In addition the fus3 and lec2 mutants cause a partial transformation of the cotyledons to leaves. In maize loss of function mutants of vivparous1 (vp1) result in ABA insensitive seeds. In the aleurone of the endosperm, vp1 mutants fail to activate C1 expression, one of the key regulators of anthocyanin synthesis genes, resulting in colorless kernels. Vp1 was cloned by transposon-tagging and encodes a plant specific B3 domain containing transcription factor (McCarty et al. 1991). VP1 plays a critical role in the dormancy to germination transition by functioning both as a transcriptional activator to regulate ABA-inducible gene expression required for dormancy and as a transcriptional repressor to repress germination-specific α-amylase gene expression (Hoecker et al. 1995). The maize Vp1 gene can complement the aba insensitive3 (abi3) mutant phenotype in Arabidopsis, which is similarly blocked in seed maturation.

Signal transduction pathways encompass two types of interaction, namely protein-protein and DNA-protein interactions. The signal transduction pathway for ABA-induced gene expression has led to the identification of a number of cis-acting DNA elements and transcriptional factors that represent both kinds of interactions. One such cis-acting element
shown to be important in ABA mediated signaling is the ABA response element (ABRE) (Yamaguchi-Shinozaki and Shinozaki 2005). ABREs, which are ACGT-containing ‘G-boxes’ in promoter elements have been shown to confer ABA inducibility to a heterologous promoter. In many promoters, a second cis-acting element, called a coupling element (CE) along with a single ABRE element is required to form an ABA-responsive complex (ABRC) (Busk and Pages 1998, Singh 1998). Two types of CEs, CE1 and CE3 together with their corresponding ABREs form ABRC1 and ABRC3 complexes, respectively (Shen and Ho 1995, Shen et al. 1996, Shen et al. 2004). Spacing requirements between the ABRE and CE for the two ABRC’s varies with 20bp for ABRC1 and 10bp for ABRC3. Identification of transactivators that bind the ABRC’s has been limited compared to the characterization of the cis regulatory elements. Transcriptional regulators that mediate ABA-induced gene expression through the ABRC complexes are VP1/ABI3, bZIP (ABI5) and APELATA2 (AP2)-like ABI4 transcriptional factors. The bZIP transcriptional factor, ABI5 has been shown to interact as dimers with ABREs (Finkelstein, Gampala and Rock 2002). VP1 has been shown to transactivate ABRC3s found in maize rab28 (Busk et al. 1997) and in barley (Busk, Jensen and Pages 1997) (Hordeum vulgare) HVA1 (Shen, Zhang and Ho 1996) but not ABRC1 in barley HVA22 (Shen et al, 1996). The only known transcriptional factor reported to bind the CE1 element has been an AP2-like protein ZmABI4, the maize ortholog of ABI4 (Niu et al. 2002). The abi4 mutant has been isolated from two independent screens, an ABA insensitivity screen similar to the one used to isolate abi3 and abi5 and a sugar-insensitivity screen (Arenas-Huertero et al. 2000, Rook et al. 2001). This suggests that ABI4 is important point in the signal transduction pathways of both ABA and sugar signaling.
VP1/ABI3 and ABA regulate the expression of both independent as well as overlapping set of genes. VP1 regulates gene transcription through both direct and indirect interactions with cis-elements. VP1 can activate the expression of genes such as maize C1, through the direct binding to Sph/RY elements via the B3 domain (Suzuki et al. 1997).

Indirect interaction of VP1 with ABREs via bZIP transcription factors like TRAB1 is seen in the case of ABA-inducible genes, such as Em (Hobo et al. 1999b). VP1 functions as a transcriptional activator for dormancy associated genes and as a transcriptional repressor of germination-related α-amylase expression in aleurone cells. Transcriptional activation functions appear to require the amino-terminal acidic domain of VP1, but repression was independent of the amino-terminal acidic domain (Hoecker, Vasil and McCarty 1995, Hoecker et al. 1999).

Our understanding at present of aleurone cell fate specification and differentiation is limited. The aleurone mutants provide an insight into the molecular switches that result in the peripheral cell attaining aleurone cell identity. The nature of these molecular switches and signaling mediated through them is still to be deciphered. CR4, a plant receptor-kinase defines an early step in aleurone cell fate and appears to be a logical candidate to understand the signal transduction pathway that finally results in aleurone cell fate specification. During the late stages of differentiation, the role of Vp1 in anthocyanin synthesis and the processes involving maturation and dormancy has been extensively studied. However, the transcriptional regulation of Vp1 has not been studied. The cis-elements ABRE and ‘CE1-like’ of the Vp1 promoter specifically bound proteins in embryo nuclear extracts, suggesting the possibility of a functional ABRC regulatory complex with trans factors regulating Vp1 transcription (Cao et al. 2007). The CR4 and Vp1 studies to date suggest the intermediate
steps between CR4 mediated cell fate specification and *Vp1* transcription are unknown. The knowledge of the genes functioning in these steps will help us better understand the molecular mechanism of aleurone cell fate specification and differentiation.

The main objective of this research was to understand the process of signal transduction and cell fate determination in aleurone cells. It includes three specific goals. (1) To identify and characterize interactors of ACR4. (2) To address the role of the interactors in dynamics of CR4 receptor function. (3) To identify transcriptional regulators of *Vp1*. To identify ACR4 interactors a two-hybrid approach with the cytoplasmic domain was used to screen a 3-day old seedling library. Chapter 2 describes the identification of CSN5 subunit of the COP9 signalosome (CSN) that interacts and regulates the accumulation of ACR4. To identify transcriptional regulators of *Vp1*, the CEIL element in the *Vp1* promoter was used to identify proteins that bind in a yeast one-hybrid screen. In Chapter 3, we describe CE1LBP1, a novel zinc binding protein that binds the CEIL element of *Vp1* promoter. Through this study, we have enhanced our understanding of cell fate specification and maturation processes that operate in the aleurone cells during cereal grain development.

**DISSERTATION ORGANIZATION**

This dissertation is organized into four chapters. Chapter one provides general background information pertaining to the study and the objectives of the research. The following two chapters represent original research, each in the form of a manuscript. The fourth chapter summarizes the conclusions from the study.

Chapter two, a journal paper being prepared for submission entitled “The COP9 signalosome (CSN) is involved in the turnover of the Arabidopsis ACR4 receptor-like
kinase” is co-authored by Antony Chettoor and Kejian Li. This chapter details the identification and the role of the COP9 signalosome (CSN) in the signaling of ACR4. Antony Chettoor performed the research and writing related to in-vivo and in planta interactions of CSN5 and ACR4 under the supervision of Philip Becraft. Kejian Li preformed research and writing related to the yeast two hybrid screen and in-vitro interactions. Xueyuan Cao generated the ACR4-GFP transgenics and statistical analysis of curcumin treatment. Robert Doyle helped in FRET analysis.

Chapter three, a journal paper being prepared for submission entitled “CE1LBP1, a novel protein binds CEIL element of the Maize Vp1 promoter”. This chapter details the characterization of a novel transacting factor, CE1LBP1 that binds the CEIL cis element of the Maize Vp1 promoter. Antony Chettoor designed, carried out the research and wrote the manuscript under the supervision of Philip Becraft.
Figure 1. Proposed genetic pathway for the regulation of aleurone cell development in maize. CR4 and DEK1 positively regulate the early steps in aleurone cell fate acquisition. Thk* defines a negative regulator of aleurone cell fate. Factors of unknown function (putative ligands) upstream of CR4 and/or DEK1 are proposed to initiate the signal transduction pathway. Nkd* and Vp1 control cell differentiation late in aleurone differentiation. VP1, in the presence of ABA, promotes aleurone maturation and dormancy by upregulating dormancy factors like LEA and repressing factors for germination such as α-amylase. VP1 along with C1 and R1 regulate the biosynthetic pathway leading to the accumulation of anthocyanin pigments. ♦: Factors (CR4 and VP1) of focus in this thesis.
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CHAPTER 2. ACR4 RECEPTOR-LIKE KINASE TURNOVER INVOLVES THE COP9 SIGNALOSOME (CSN)

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ABSTRACT

The ability to perceive and transduce signals in a range of plant responses is mediated through receptor kinases. \textsc{Arabidopsis crinkly4} (ACR4) is unique among known plant receptor kinases because functional receptors appear to rapidly turnover. In this study, we identified CSN5 subunits of the COP9 signalosome (CSN) as interactors of cytoplasmic domain of ACR4. \textit{In vitro} interactions confirmed that CSN5 subunits bind and are phosphorylated by ACR4. \textit{In vivo} FRET experiments demonstrated that ACR4 was in close proximity to CSN5 within the context of a plant cell. \textit{In planta} evidence was demonstrated by the accumulation of ACR4-GFP in transgenic plants treated with curcumin, a COP9 signalosome inhibitor. In addition, ACR4-GFP accumulated in a \textit{csn5} double mutant background. Thus, the COP9 signalosome appears to be involved in the turnover of ACR4.

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INTRODUCTION

Cells communicate with each other and with the environment by signaling through cell surface receptors. One class of cell surface receptors called receptor kinases is defined structurally by the presence of an extracellular domain that often communicates to the outside by binding a ligand, a membrane spanning domain and a cytoplasmic kinase domain. Plant receptor-like kinases (RLKs) are involved in hormonal response pathways, cell differentiation, plant growth and development, self-incompatibility, and pathogen recognition (Afzal et al. 2008, Becraft 2002, Hematy and Hofte 2008, Morris and Walker 2003, Tichtinsky et al. 2003)

The maize (Zea mays) CRINKLY4 (CR4) gene encodes a receptor-like kinase that controls a variety of cell differentiation responses (Becraft et al. 2001, Becraft et al. 1996, Jin et al. 2000). Mutants in the maize cr4 gene disrupt aleurone cell fate specification causing peripheral endosperm cells to be replaced by starch cells. The mutant plants also appear short with leaves crinkled and fused (Becraft et al. 1996). CR4 encodes a 901-amino acid RLK with an extracellular domain containing seven copies of a 39-amino acid repeat termed “crinkly repeats” that are predicted to fold into a β-propeller structure and a domain that resembles the extracellular domain of mammalian tumor necrosis factor receptor (TNFR). The intracellular domain consists of a serine/threonine kinase domain, a 40-amino acid juxtamembrane region and a 116-amino acid carboxyl-terminal domain of unknown function (Becraft et al. 1996). In the Arabidopsis genome, out of the 417 or more predicted RLKs, five are predicted to encode CR4-related genes. One of the five, designated as ACR4 contains all the features of the maize CR4 and is believed to be the Arabidopsis ortholog (Cao et al. 2005, Gifford et al. 2003, Tanaka et al. 2002, Watanabe et al. 2004). ACR4
mutants unlike CR4, do not exhibit any gross defects in plant morphology, but show abnormalities in seed morphology and seed set. ACR4 is required during normal development of ovule, integument and sepal margins (Gifford et al. 2003). Subtle changes in cell shape and cuticle formation indicate that ACR4 is required for normal leaf differentiation (Watanabe et al. 2004). In roots, ACR4 regulates both apical root and lateral root initiation by controlling the number and plane of formative divisions during organogenesis (De Smet et al. 2008).

Stereotypical receptor kinase mediated signaling in animals involves ligand binding, receptor oligomerization, phosphorylation of the cytoplasmic domain and recruitment of interactors that bind phosphorylated residues of the receptor (Schlessinger and Ullrich 1992, Ullrich et al. 1984). The epidermal growth factor (EGF) and its cognate receptor epidermal growth factor receptor (EGFR) is the best characterized ligand/receptor signaling pathway. Upon ligand binding, the EGF/EGFR complex is endocytosed and then the receptor either recycled or targeted for degradation (Jiang and Sorkin 2003, Maxfield and McGraw 2004, Sorkin and Goh 2008, van der Knaap et al. 1999, Wang and Moran 1996, Wiley et al. 1991).

In contrast, knowledge about receptor signaling in plants is limited. Of the ligand-receptor pairs known in plants, BRASSINOSTEROID INSENSITIVE 1(BRI1) which binds brassinosteroid (BR) is the best characterized (Gendron and Wang 2007, Kinoshita et al. 2005). Geldner et al (2007) found functional BRI1/BAK1 (BRI1- associated kinase 1) complexes in endosomes whereas nonfunctional BRI1/BKI1 (BRI1 kinase inhibitor1) complexes were restricted to the plasmamembrane. BRI1 trafficking is constitutive and the endosomal pool is functional in signal transduction (Geldner et al. 2007).
Turnover of receptors and other membrane proteins depend on two processes, namely endocytosis and/or ubiquitin-mediated protein degradation. Monoubiquitination serves as a signal for endocytosis, whereas polyubiquitination has been associated mainly with targeting substrates to the proteosome (Haglund et al. 2003). Ubiquitination of proteins is catalyzed by the action of an E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzymes and E3 ubiquitin ligase. E3 ubiquitin ligase can function as a single peptide (Mdm2 and XIAP) or as a multiple component complex (Skp1-Cullin-F-box protein [SCF]) (Bosu and Kipreos 2008). In multiple component E3 ligases, the Cullin subunit is the scaffold which links a substrate-binding adaptor with an E2-binding RING. The adaptor protein links the substrate recognition protein to the cullin. Covalent attachment of the ubiquitin-like protein NEDD8 to the cullin subunit stimulates E3 ligase ubiquitination activity (Bosu and Kipreos 2008, Hotton and Callis 2008).

The COP9 signalosome (CSN) is a conserved protein complex that functions in the ubiquitin-proteosome pathway. The CSN was originally identified by mutants that exhibited constitutive photomorphogenesis and pigmented seed-coats, but has been shown to be present in many organisms and perform diverse functions (Wei and Deng 1992). The best characterized biochemical activity that can be ascribed directly to CSN is the isopeptidase activity that removes the NEDD8 attachment of the culllin subunit of the cullin-E3 ligases (Cope et al. 2002). The CSN complex is made up of eight subunits designated CSN1- CSN8. The CSN5 subunit is unique in that it harbors the catalytic center for deneddylation activity and can also stably exist independently of the CSN holocomplex in-vivo (Wei and Deng 2003). CSN5 is encoded by a single gene in most organisms, except in Arabidopsis it is encoded by two highly homologous, partially redundant genes CSN5A and CSN5B.
(Gusmaroli et al. 2004). The complete loss of CSN5B does not result in any obvious developmental defect, whereas the loss of CSN5A triggers a number of pleiotrophic developmental defects. Double null homozygous mutants invariably die at the seedling stage, virtually identical to the null alleles of the cop/det/fus mutants, and do not express detectable CSN5 proteins (Gusmaroli et al. 2007).

ACR4 localizes to the plasmamembrane and endosomal vesicles similar to BRI1 RLK (Geldner et al. 2007, Gifford et al. 2005, Gusmaroli et al. 2004). Using lines expressing ACR4-GFP fusions under native promoters, Gifford and colleagues observed fluorescence in the plasmamembrane and endosomal vesicles. Thus, it is possible that ACR4 receptor turnover is regulated by ubiquitin-mediated protein degradation. The aim of this study was to identify intracellular components capable of interacting with the cytoplasmic domain of ACR4. Among the interacting proteins identified was CSN5 subunit. We show that ACR4 associates with CSN5 in plant cells and that the CSN is involved in the turnover of ACR4.

RESULTS

Identification of proteins that interact with the cytoplasmic domain of ACR4

To identify other components of the ACR4 signal transduction system, we performed a yeast two-hybrid screen to identify putative interactors with the ACR4 cytoplasmic domain. The region encoding the ACR4 cytoplasmic domain was cloned into the bait vector pGBD-C1 (James et al. 1996) to produce an in-frame fusion with the GAL4 DNA-binding domain (pGBD-CR4). This construct was used to screen an Arabidopsis 3 day-old seedling cDNA library in the yeast YRG2 strain. From screening approximately one million yeast transformants, ninety four colonies grew on the SD/-Trp/-Leu/-His selective medium
supplemented with 5mM 3-AT and turned blue in the colony-lift filter assay for lacZ expression. The relative strength of these interactions was measured using the ONPG (o-nitrophenyl β-D-galactopyranoside) liquid culture assay for β-galactosidase activity (data not shown). The fourteen clones that showed the strongest interactions were chosen for further study. Sequence analysis revealed that these represented six independent clones. One gene was not expressed in the same tissues as ACR4 and was not pursued further. None of these prey clones showed positive interactions in combination with the empty pGBD-C1 vector, indicating the interactions required the ACR4 cytoplasmic domain (data not shown).

Two of the clones indentified as CSN5A (COP9SIGNALOSOME 5A subunit; At1g22920) and CSN5B (COP9SIGNALOSOME 5B subunit; At1g71230) were studied further because of the known role of the COP9 SIGNALOSOME (CSN) in protein turnover. CSN5A and CSN5B encode two closely related proteins that function as subunits of the CSN (Dohmann et al. 2005, Gusmaroli et al. 2004, Jin et al. 2000, Kwok et al. 1998). CSN5 proteins appear to be ubiquitously expressed in various Arabidopsis organs, including seedlings, siliques, flowers, leaves, stems and roots, with higher expression in floral tissues and lower expression in siliques and leaves (Kwok et al. 1998). ACR4 is expressed in various tissues with the strongest expression in shoot apical meristems (SAM) and flower buds. The overlapping expression patterns allow the possibility that CSN5 proteins interact with ACR4 in the plant. The more extensive expression pattern of CSN5 is consistent with the CSN5 proteins functioning in additional pathways, such as photomorphogenesis.
The interactions between ACR4 and CSN5B do not require ACR4 kinase activity

To verify the interactions between ACR4 and these identified proteins, an *in vitro* pull-down assay was carried out using *E. coli* expressed proteins. The cytoplasmic domain of ACR4 was cloned into a pET-32a (+) expression vector to create a fusion protein (ACR4K) tagged with the thioredoxin/6X His/S-tag (THS). The THS alone was also expressed in *E. coli* and used as a negative control in the pull-down assay. The same regions of the interacting proteins as those identified in the yeast two-hybrid screen were cloned into pGEX-5X1 (Amersham Pharmacia Biotech) to make GST fusion proteins, and a c-myc tag was added to the carboxyl end of each protein. ACR4K was incubated with each putative interacting protein, pulled down using TALON resin (Clontech) to bind the 6X His tag and the presence of the interacting proteins detected using anti-myc antibody. As shown in Figure 1, none of the fusion proteins interact with the control THS tag, while CSN5B binds with ACR4K. However, CSN5A did not show a detectable level of binding under these conditions.

Ligand binding typically induces phosphorylation of the cytoplasmic domain of receptor kinases, and interactions with downstream factors are often phosphorylation dependent. In this way, signal transduction components are recruited to an activated receptor complex. To test whether the interactions between the cytoplasmic domain of ACR4 and the interacting proteins require ACR4 kinase activity, an *in vitro* binding assay was carried out using a kinase-dead ACR4. The essential Lys540 of ACR4 kinase domain was replaced by Ala to create an inactive kinase (ACR4KM). ACR4KM does not autophosphorylate, indicating that the substitution abolished kinase activity (Cao *et al*. 2005). As shown in Figure 1, CSN5B was pulled down with the kinase-dead ACR4 at about the same level as
with the active ACR4. Thus, the interactions between ACR4 and CSN5B do not require ACR4 kinase activity. Like with the kinase active ACR4, CSN5A did not show binding with the kinase-dead ACR4.

**Phosphorylation of the interacting proteins by ACR4**

A common mechanism in the regulation of signal transduction systems is for receptor kinases to regulate downstream components through phosphorylation. Previous work has shown that ACR4 is an active serine/threonine kinase (Cao et al. 2005, Gifford et al. 2003). To test whether ACR4 can phosphorylate the interacting proteins, we conducted a phosphorylation assay. Because the sizes of these interacting proteins were similar to the two strong ACR4K autophosphorylation bands, we covalently bound ACR4K to NHS-activated sepharose beads. The interacting proteins were mixed with the ACR4K-coupled sepharose in kinase buffer containing $[^\gamma\text{-}32\text{P}]$-ATP. After incubation, the sepharose was pelleted by centrifugation and the supernatants applied to an SDS-PAGE gel shown in Figure 2A and 2B. The pellets were resuspended in SDS loading buffer, boiled, and the supernatants containing the released interacting proteins loaded onto another SDS-PAGE gel (Figure 2C, D). ACR4K did not appear in either fraction because it remained covalently bound to the sepharose.

The inactive kinase ACR4KM, which can be phosphorylated by the active kinase ACR4K (Cao et al. 2005), was used as a substrate in the same kinase assay as a positive control. As shown in Figure 2C, CSN5A and CSN5B show a single clear phosphorylated band by ACR4K. None of the interacting proteins showed phosphorylation by ACR4KM (data not shown), indicating that phosphorylation of the interacting proteins was catalyzed by
ACR4K. Thus, CSN5A and CSN5B can be phosphorylated in vitro by ACR4K indicating that they are potential substrates of ACR4.

**Binding to ACR4 is independent of the phosphorylation state of the interactors**

Phospho-regulation of downstream factors is critical in many receptor kinase signal transduction systems. Phosphorylation might regulate activity, turnover, cellular translocation or further protein associations. As such, it is conceivable that phosphorylation of the interacting proteins could promote dissociation with ACR4. To test this, we compared the supernatants and pellet fractions from the phosphorylation assay. ACR4-bound proteins appeared in the pellet fraction, while unbound proteins appeared in the supernatants. As shown in Figure 2C, the phosphorylated CSN5B remained bound to ACR4, indicating it had not dissociated after phosphorylation. In contrast, part of the phosphorylated CSN5A appeared in the supernatant, suggesting that phosphorylation of CSN5A might cause some disruption of the interaction, allowing part of the phosphorylated proteins to dissociate from ACR4K, or that CSN5A binding to ACR4 is weak regardless of the phosphorylation status.

**The carboxyl-terminal domain of ACR4 interacts with both CSN5A and CSN5B proteins**

The cytoplasmic domain of ACR4 consists of three domains: the juxtamembrane region, the kinase domain, and a 116 amino acid carboxyl-terminal domain of unknown function. The complete cytoplasmic domain was used as bait in the two-hybrid screen. To test which domains interact with CSN5A and CSN5B, we separately cloned the kinase domain (BD-KD) and the carboxyl-terminal domain (BD-CT) into the yeast two-hybrid bait vector pGBD-C1 (James et al. 1996). The results are shown in Table 1. CSN5A showed
interactions with both BD-KD and BD-CT, whereas CSN5B interacted only with BD-CT but not with BD-KD. The interactions were confirmed by lacZ reporter gene expression in a colony-lift filter assay (Table 1). Thus, the kinase domain or the carboxyl-terminal domains of the ACR4 are sufficient for the interactions with CSN5A, whereas the carboxyl-terminal domain of ACR4, but not the kinase domain, is necessary and sufficient, for the interactions with CSN5B.

It was surprising that CSN5A interacted with both the kinase and carboxyl-terminal domains. We searched for motifs present in both the kinase and carboxyl-terminal domains that could be responsible for these interactions. As shown in Figure 3, a potential motif was found in both domains. The motif consists of SS(X)_9G(X)_6DE(X)_2K(X)_3A(X)_5EE(X)_3A (where X stands for any amino acid) where the relative spacing of SS, G, DE, K, A and EE are conserved. There is also an SEN before SS, and an SA between SS and G with variable spacing. This opens up the intriguing possibility that this motif could be responsible for the interactions with the proteins that recognized both the kinase and carboxyl-terminal domains of ACR4.

**Förster resonance energy transfer (FRET) indicates ACR4 interacts with CSN5A and CSN5B in vivo**

FRET donor fluorescence recovery following acceptor photobleaching was used to test the interactions between CSN5A, CSN5B and ACR4 within the context of plant cells. The donor fusion protein ACR4-CFP was co-expressed with each acceptor fusion CSN5A-YFP and CSN5B-YFP in onion epidermal cells. FRET was quantified as donor dequenching after photobleaching of the acceptor (Miyawaki and Tsein, 2000). For each donor and
acceptor fusion pair, FRET occurrence is shown by the difference in donor signal intensity between acceptor pre-bleach and post-bleach. The basis for this is that energy that had been transferred from the donor to the acceptor before acceptor photobleaching will be emitted as increased donor fluorescence following acceptor bleaching. As controls, ACR4-CFP was expressed alone, co-expressed with YFP, and YFP was expressed alone in onion epidermal cells. As seen in Figure 4C and G, FRET did occur for ACR4-CFP/CSN5A-YFP and ACR4-CFP/CSN5B-YFP fusion pairs as shown by the difference in CFP signal intensity between acceptor prebleach and post bleach. FRET was not observed in the case of ACR4-CFP/YFP pairs that served as negative controls (Figure 4K). Onion epidermal cells transiently transformed with the ACR4-CFP or YFP alone did not result in any observable FRET (Figure 4O and S). Thus, FRET confirms that ACR4 is in close proximity to both CSN5A and CSN5B within a plant cell.

Curcumin treatment, a CSN inhibitor prevents ACR4 turnover

Sequence analyses indicate that CSN5A and CSN5B share 86 and 88% identity at the nucleotide (cDNA) and amino acid levels, respectively. The fact that yeast two hybrid screen identified both CSN5A and CNS5B subunits of the CSN complex to interact with ACR4 suggests a role for the CSN-ubiquitin proteosome system. Since ACR4 protein has b (Gifford et al., 2005), we hypothesized CSN might be involved in ACR4 turnover. This was tested pharmacologically using the CSN inhibitor curcumin. Curcumin (diferuloylmethane) is a natural polyphenolic compound extracted from the spice turmeric (Curcuma longa) that has been reported to have antioxidant, anti-inflammatory, and antiproliferative properties (Dutta et al. 2005, Quiles et al. 2002). It has also been well documented in mammalian systems that
curcumin is an inhibitor of the COP9 signalosome through the inhibition of its associated kinases, such as CKII and PKD (Chaudhary and Hruska 2003, Uhle et al. 2003). To test whether CSN was involved in ACR4 turnover, we treated ACR4-GFP seedlings with 10µM curcumin and monitored GFP accumulation in roots using confocal microscopy. Mock treatments of ACR4-GFP and wild type plants, or curcumin treated wild type plants, were used as controls. Confocal image stacks across the whole root were collected from all samples and signal intensities quantified. The results are shown in Figure 5. Curcumin treatment of ACR4-GFP plants is significantly different from ACR4-GFP plants treated with DMSO alone. The increase in fluorescence intensity of the ACR4-GFP plants treated with curcumin was significant compared to wild type curcumin treatment (p<0.001) (Figure 5E). Wild type controls showed no difference in the presence or absence of curcumin. Thus, the results suggest that inhibition of the COP9 signalosome by curcumin inhibited ACR4 protein turnover.

**ACR4 accumulates in csn5a-1 csn5b-1 double mutants**

A known biochemical role attributed to CSN is a metalloprotease activity responsible for the de neddylation of cullins, core components of the multiple component RING types of ubiquitin E3 ligases (Lyapina et al. 2001, Zhou et al. 2001). To test for a role of the COP9 signalosome in ACR4 turnover, csn5a-1/+; csn5b-1/ csn5b-1 plants were crossed to ACR4-GFP plants and the F2 progeny seedlings were screened for phenotypes similar to cop/det/fus. Figure 6 shows that the ACR4-GFP csn5a-1 csn5b-1 plants produced more GFP fluorescence than ACR4-GFP controls. Only background fluorescence was detected in csn5a-1 csn5b double mutant seedlings and wild type seedlings. Thus, ACR4-GFP
accumulated to higher levels in the absence of CSN5 function, supporting the role of the CSN5 subunits of the COP9 signalosome in the turnover of ACR4.

DISCUSSION

ACR4 interactions with CSN5

ACR4, a developmental RLK, is structurally different from the predominantly LRR type of RLKs characterized in plants. Identification of ligands and other components of the ACR4 signal transduction pathway is critical to our understanding of receptor signaling in plants. We utilized the yeast-two hybrid approach to identify interactors with the ACR4 cytoplasmic domain and recovered CSN5A and CSN5B. The fact that both isoforms of CSN5 were picked up in the screen suggests that these proteins are possible bonafide interactors of ACR4.

ACR4 shows the highest level of expression in the shoot apical meristem and organ primordia (Cao et al. 2004, Gifford et al. 2003, Tanaka et al. 2002). The CSN5 genes are ubiquitously expressed in various tissues with high expression in floral and root tissues. The expression profile of CSN5 coincides with regions of highest ACR4 expression, consistent with the possibility that these proteins could interact with ACR4 in the plant.

The in vitro interaction of CSN5B was confirmed by pull-down (Figures 1, 2). The interaction did not require ACR4 kinase activity as the interaction occurred even with a kinase inactive mutant form of ACR4. In contrast, the pull-downs with CSN5A were only successful in the phosphorylation assay. The basis for the discrepancy between the two pull down experiments is not clear but one possibility is that phosphorylation is required to stabilize the interaction, since ATP was present in the kinase assay and in yeast cells but was
not supplied for the pull down reactions. In mammalian EGFR signaling, in keratinocytes upon UV irradiation EGFR activation results in tyrosine phosphorylation of β-catenin. β-catenin, a cadherin protein complex subunit relocates from the membrane to the nucleus and regulates gene expression (Zhang et al. 2001). Experiments were conducted to test the ability of the ACR4 kinase to phosphorylate CSN5A and CSN5B, which could result in the recruitment of additional factors to a signaling complex or potentially could cause dissociation as a prelude to subcellular translocation or other response of the interacting protein. Our results showed that ACR4K could phosphorylate CSN5A and CSN5B in vitro. In the case of CSN5B, the phosphorylated protein remained bound to ACR4, while phosphorylated CSN5A was observed in both the bound and unbound fractions. At this point it is unclear whether phosphorylation destabilized the interaction or whether the dissociated CSN5A reflects an intrinsically weak interaction. The basis of the observed differences between the two CSN5 isoforms and their functional implications need further testing.

FRET experiments confirmed the interactions of both CSN5 isoforms with ACR4 in onion epidermal cells. The absences of FRET in the ACR4CFP/YFP and ACR4CFP along with the robustness of the acceptor photobleaching method confirm the authenticity of the ACR4/CSN5 interactions. CSN5 has been found to be present as monomers, small complexes and large holocomplexes with the other seven CSN subunits of the CSN complex in-vivo (Wei and Deng 2003). The locations of these varied forms of CSN5 complexes have not been characterized to date. The CSN5 subunits are cytoplasmic and nuclear localized, while ACR4 is localized to plasmamembrane and endosomes (Gifford et al. 2005, Kwok et al. 1998, Watanabe et al. 2004). This suggests the interactions occur either at the
plasmamembrane and/or in the cytoplasm. Taken together, it is possible that ACR4 is processed prior to interaction with CSN5 subunits. This possibility remains to be tested.

**ACR4 receptor turnover**

The lack of detectable functional ACR4 is attributed to its rapid turnover. It has been proposed that ACR4 in *Arabidopsis* root cells was present in two distinct subcellular compartments: protein export bodies or secretory vesicles and a population of internalized vesicles or endosomes (Gifford *et al.* 2005, Wang and Moran 1996). Furthermore, internalization and turnover for ACR4 appear to be linked, operating through endosomes. In maize, CRINKLY4 (CR4), DEFECTIVE KERNEL1 (DEK1) and SUPERNUMERARY ALEURONE LAYERS1 (SAL1), involved in aluerone cell fate specification, colocalize in endosomal compartments (Tian *et al.* 2007). Taken together these studies suggest that the endosomal internalization and turnover of ACR4 are possibly linked.

ACR4 shares some similarities with the LRR type BRI1 receptor kinase. Both colocalize with the endocytic tracer FM4-64 and show sensitivity to brefeldin A (BFA) treatment. Thus, ACR4 might represent another example of a receptor exhibiting constitutive endocytosis. However, the ligand for ACR4 has not been identified and therefore it is possible that continuous ligand stimulation results in endosomal localization. The ACR4 receptors differ from BRI1 receptor in protein stability. It is possible that the interaction of ACR4 with CSN5 of the CSN complex might result in rapid turnover. The most well characterized function of CSN is the regulation of protein degradation which involves the removal of NEDD8 from cullin-based E3 ubiquitin ligase. Two experiments support the role CSN in ACR4 receptor turnover. (1) *Arabidopsis* plants expressing ACR4-GFP fusion protein treated with curcumin, a specific inhibitor of the CSN complex, displayed increased
amounts of ACR4-GFP accumulation. (2) In-plant accumulation of ACR4-GFP fusion protein in csn5a csn5b double mutant background. It is likely that the CSN functions in ubiquitin-mediated protein degradation of the receptor, resulting in the rapid turnover of the receptor and the corresponding inability to detect functional ACR4 protein. In the case of BRII1 the absence of rapid turnover and/or more receptor recycling accounts for the detection of full length BRII1.

There are several examples in animal systems where the CSN5 subunit functions in receptor turnover or membrane protein regulation. CSN5 coimmunoprecipitates with the nuclear estrogen receptor α (ERα) and overexpression of CSN5 results in ligand-induced ERα degradation (Callige et al. 2005). CSN5 can also bind to the misfolded cystic fibrosis transmembrane conductance regulator (CFTR), a membrane protein, and target it to the degradative pathway (Tanguy et al. 2008). In COS7 cells, CSN5 interacts with, and inhibits the activity of, the membrane localized cardiac L-type Ca^{2+} channel (Kameda et al. 2006).

In summary, the CSN5 subunit of the COP9 signalosome complex appears critical to the rapid turnover of the ACR4 receptor. The fact that the CSN5 interactions identified here have not been reported in other RLK studies suggests the signal transduction pathway for ACR4 could be novel. Future work will examine the function of CSN-mediated ACR4 turnover in signaling and decipher in greater detail the ACR4 signal transduction pathway.

**METHODS**

**Plant Materials and Growth Conditions**

The wild-type *Arabidopsis thaliana* plants used in this study are Columbia-0 ecotype. The CSN5 T-DNA insertion lines SALK_063436 (csn5a-1) and SALK_007134 (csn5b-1)
were identified in the SIGNAL database and obtained from ABRC (Alonso et al. 2003). *csn5a-1, csn5b-1* single mutants and *csn5a-1 csn5b-1* double mutants have been characterized as null CSN5 mutants (Gusmaroli et al. 2007). The Arabidopsis seeds were surface sterilized and the plants grown on solid 1× MS medium supplemented with 1% sucrose or on a mixture of 1:1 soil: vermiculite under long-day conditions (16 h light/8 h dark) in a controlled environment chamber at 22°C.

**Construction of ACR4-GFP Transgenics**

A 1.38 kb promoter region upstream of the ACR4 start codon was amplified from pBFScX by PCR (Cao et al., 2005). The PCR fragment was cloned into SmaI/BamHI site of the vector pEZS-NL (a gift from D. Ehrhardt, Dept of Plant Biology, Stanford) resulting in pBFSP EZ. The ACR4 ORF was amplified and cloned into pBFSP EZ as an Eco47III/BamHI fragment resulting in pACR4EZ. The ACR4-eGFP chimera was cut out as a SacI/SalI fragment and cloned into binary vector pCAMBIA2300 to obtain a translational fusion of the ACR4 and eGFP coding sequence driven by the ACR4 native promoter. The construct was introduced into *Agrobacterium tumefacians* strain C58C1. Plants were transformed by the floral-dip method modified from the vacuum-infiltration method (Bechtold and Pelletier 1998). Seeds from the infiltrated plants were harvested, surface-sterilized and sown on MS plates supplemented with 30 µg ml⁻¹ hygromycin. Hygromycin-resistant seedling (T1) were transplanted into soil, grown to maturity and seeds harvested.

**Yeast two-hybrid library screening**

pGBD-C1 (James et al. 1996), a GAL4 DNA-binding domain vector, was used to construct the bait plasmid. The DNA fragment encoding the cytoplasmic domain of ACR4
was inserted to generate an in-frame fusion between the DNA-binding domain of GAL4 and the cytoplasmic domain of ACR4, including amino acids 458-895 (pGBD-ACR4). An *Arabidopsis* λACT cDNA library from 3 day-old seedlings was obtained from the *Arabidopsis* Biological Resource Center (stock number CD4-22) of the Ohio State University and used as the prey in the yeast two hybrid screening. The library was converted to plasmid and the plasmid DNA prepared using a Qiagen plasmid Maxi kit.

The yeast host strain YRG2 (Stratagene) was transformed with plasmid DNA from the library. Approximately 1 million transformants were plated on synthetic dextrose (SD) minimal medium with 5mM 3-amino-1,2,4-triazole (3-AT) and lacking Trp, Leu and His. Those that grew were subjected to the colony-lift β-galactosidase filter assay for *lacZ* expression. The positive colonies were then streaked on the SD/-Leu plates with 0.5g/l 5-FAA (5-fluoroanthranilic acid) after subculture three times in liquid SD/-Leu media to rescue the library (prey) plasmid. The library plasmids were isolated and to verify positive interactions, the library plasmid was retransformed back into yeast YRG2 strain in combination with either pGBD-ACR4, or empty pGBD-C1 vector, and plated on SD/-His/-Trp/-Leu containing 5mM 3-AT.

**DNA sequence analysis**

The cDNA inserts of the positive colonies were sequenced at the DNA Facility at Iowa State University. DNA sequences were used in database searches by BLAST (Altschul *et al.* 1997). Cellular localizations of encoded proteins were predicted using PSORT (Nakai and Horton 1999) (http://psort.nibb.ac.jp) and SignalP (Nielsen *et al.* 1997) (www.cbs.dtu.dk/services/SignalP).
Protein expression

The cytoplasmic domain of ACR4 (Arg458-Phe895) was cloned in the pET-32a (+) vector (Novagen) with Trx/6X His/S-Tag (THS) and expressed in *E. coli*. Expression of this protein, ACR4K, was induced in 1mM IPTG (Sigma) and proteins were purified from the cell lysate using TALON purification kit (Clontech). As a control, the THS tag was expressed and purified from the empty pET-32a (+) vector using the same procedure as above. A kinase-dead ACR4 (ACR4KM) was also expressed in *E. coli* by substituting the essential Lys540 with Ala. The mutation was introduced by PCR where the AAG to GCG codon change was incorporated by primer mismatch.

Pull-down assays

Approximately 5µg of purified ACR4K, ACR4KM, or THS (the fusion peptide consisting of thioredoxin-, S- and 6X His tags encoded by the empty PET vector) protein was combined with approximately 5µg purified CSN5A and CSN5B protein in 200µl binding buffer [50mM Tris pH 8.8, 300mM NaCl, 50mM sodium phosphate, and DOC (1.5% for interactions with CSN5B and phosphatase, 7% for interactions with CSN5A, CIRK1 and CIRK2)]. 10µl TALON resin (Clontech) was added with continued rocking at room temperature for 45 additional minutes. Beads were then pelleted by brief centrifugation and washed 6 times with 200µl binding buffer. 20µl 1x SDS loading buffer was added to each reaction and the resuspended beads were boiled for 5 minutes. After brief centrifugation, the supernatants were loaded on a 10% SDS-PAGE gel, electrophoresed and transferred to nitrocellulose membrane. The membrane was immunoblotted with the anti-myc primary antibody (Invitrogen, 1:5,000 dilution) and then with the goat anti-mouse IgG secondary
antibody conjugated with horseradish peroxidase (Pierce, 1:10,000). Finally, the membrane was incubated with the ECL western blotting detection reagents and analysis system (Amersham Bioscience), and exposed to Kodak X-Omat film.

**Kinase assays**

ACR4K purified protein was coupled to NHS-activated Sepharose 4 Fast Flow (Pharmacia Biotech) according to the manufacturers recommendations. Approximately 3µg NHS-activated sepharose-bound ACR4K was combined with approximately 5µg CSN5A, CSN5B, or ACR4KM in 60µl kinase buffer (50mM Tris-HCl [pH 7.5], 5mM MgCl₂, and 5mM MnCl₂). 1µl [γ-³²] P-ATP was added to each and the reaction was incubated at room temperature for 45 minutes. After brief centrifugation, the supernatants were loaded on a 10% SDS-PAGE gel. 20µl of 1x SDS loading buffer was added to the remaining beads and the resuspended beads were boiled for 5 minutes. After brief centrifugation, the supernatants from the pelleted beads were also loaded on a 10% SDS-PAGE gel. The gels were then stained with Coomassie blue, dried, and subjected to phosphoimaging (Molecular Dynamics, Model 400A) to detect the phosphoproteins.

**FRET detection by confocal microscopy**

ACR4 ORF was cloned into pECFP (BD Biosciences) as a BamHI and KpnI fragment amplified from genomic DNA obtained using the following primers: ACR4FRET and ACR4FRETB (Table 2), resulting in pACFP. The ACR4CFP fragment from pACFP was introduced into the vector pJ4GFP-XB (Igarashi et al. 2001) as BamHI/NotI fragment so that the chimeric gene replaced the original GFP, resulting in pJACFP. CSN5A and CSN5B
were amplified using cDNA’s U87212 and U13455 obtained from ABRC with the following primers: CSN5A (CSN5AYFPF and CSN5AYFPB; Table 2) and CSN5B (CSN5BYFPF and CSN5BYFPB; Table 2). The CSN5A ORF was cloned into pEYFP (BD Biosciences) as a SalI and BamHI fragment, the CSN5B ORF was cloned into pEYFP as a SalI and AgeI fragment resulting in pC5AYFP and pC5BYFP respectively. CSN5AYFP and CSN5BYFP fragments from pC5AYFP and pC5BYFP were introduced into the vector pJ4GFP-XB as SalI and NotI fragments resulting in pJC5AYFP and pJC5BYFP and respectively.

Cells from onion epidermis were placed on agar plates containing half-strength Murashige and Skoog (MS) salts and were transformed biolistically. The epidermal tissues were incubated for 20hrs at room temperature prior to analysis. Onion epidermal cells were examined for the localization of the CFP/YFP fused proteins using a fluorescence microscope (Prairie Technologies) under a filter set (all filters from Omega Optical) for CFP: Excitation filter of 440/21nm, a dichoric beam splitter of 455nm and emission filter of 480/30nm. YFP was viewed under a filter set with an excitation filter of 500/25nm, a dichroic beam splitter of 525nm, and an emission filter of 545/35nm. Images were captured using a cooled CCD camera (Hamamatsu Photonics) and controlled by Prairie View (Prairie Technologies). CFP and YFP derived fluorescence were recorded, averaged and used for corrections. To quantify the efficiency of FRET in absolute terms, we selectively photobleached the acceptor fluorophore and measured the dequenching of the donor fluorescence (Miyawaki and Tsien 2000). FRET efficiency E is given by: 

$$E = 1 - \frac{F_{da}}{F_d}$$

where $F_{da}$ is the donor emission when both donor and acceptor are present, and $F_d$ is donor emission in the absence of acceptor, (i.e. after photobleach). Three successive digital images (with appropriate background subtractions) were acquired: Pre-bleached donor, Pre-bleached
acceptor and post-bleached donor. We routinely photobleached YFP by illuminating the cells for 3–5 min with no neutral density filters. This treatment had no effect on CFP in the absence of YFP, as shown in Figure 6.

**Curcumin experiments**

ACR4-GFP young seedlings were incubated in 10µM curcumin (C 1386, Sigma-Aldrich) at 22°C. The working curcumin solution was made by diluting a 10 mM DMSO stock 1:1000 in water. Control roots were incubated for the same period of time in a 1:1000 dilution of DMSO in water and observed under a laser confocal microscope (Prairie Technologies). Confocal image stacks were collected and then processed using Metamorph 6.0 (Molecular Devices). Statistical two-way analysis of variance was performed using the SAS system (SAS Institute). Tukey’s studentized range (honestly significant difference) test was used to determine significant differences among genotypes and treatments. An α level of 0.05 was used as the criterion of statistical significance.

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monomeric form and in the COP9 complex, and their abundance is differentially

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**FIGURE LEGENDS**

**Figure 1.** The cytoplasmic domain of ACR4 binds to the interacting proteins in a pull-down assay and the interactions do not require ACR4 kinase activity.

Each interacting protein was mixed with the THS tag encoded by the empty pET-32a (+) vector (THS), the kinase-active ACR4 cytoplasmic domain (ACR4K) or the kinase-inactive ACR4 cytoplasmic domain (ACR4KM). THS, ACR4K or ACR4KM were pulled-down with TALON affinity resin, and the myc-tagged interacting proteins were detected by immunoblot with anti-myc antibody.

**Figure 2.** Phosphorylation of the interacting proteins by ACR4K.

ACR4K-coupled sepharose was mixed with each interacting protein or the kinase-inactive ACR4KM in kinase buffer containing $\alpha$-$^{32}$P-ATP.

(A, B) After centrifugation, supernatants were run on SDS-PAGE.

(C, D) Pellets containing the sepharose coupled ACR4K were boiled in SDS loading buffer and the bound proteins separated by SDS-PAGE.

(A, C) Autoradiographs.

(B, D) Coomassie blue stained gels.
Figure 3. A sequence motif found repeated in the kinase domain and carboxy terminal domain of ACR4.

Figure 4. ACR4 interacts with CSN5A and CSN5B in FRET Assays

Onion epidermal cells were transiently transformed with the ACR4 donor (CFP fusion) and interactor acceptors (YFP fusions). FRET was detected by the acceptor photobleaching technique (Miyawaki and Tsein, 2000).

(A, B, C, D) ACR4CFP+CSN5AYFP FRET pairs.
(E, F, G, H) ACR4CFP+CSN5BYFP FRET pairs.
(I, J, K, L) ACR4CFP+YFP FRET pairs.
(M, N, O, P) ACR4CFP control.
(Q, R, S, T) YFP control

(A, E, I, M, Q) Pre-bleach donor images.
(B, F, J, N, R) Post-bleach donor.
(C, G, K, O, S) FRET intensity difference profiles.
(D, H, L, P, T) Pre-bleach acceptor. Scale bar 25µm.

Figure 5. ACR4 accumulates with Curcumin, a COP9 signalosome inhibitor

ACR4-GFP transgenic and wild type Columbia seedlings were treated with 10µM curcumin or mock treated with DMSO and the sub-apical region of roots observed. Confocal image stacks were collected.

(A) Representative mid plane image of control Columbia roots with DMSO
(B) Representative mid plane image of Columbia roots with curcumin
(C) Representative mid plane image of ACR4-GFP roots with DMSO

(D) Representative mid plane image of ACR4-GFP roots with curcumin

(E) Fluorescence signal intensities was quantified (Metamorph) from image stacks and tested for statistical significance. Error bars indicate Standard error (n=10). **: Indicates significant increase in fluorescence intensity for curcumin treated ACR4-GFP roots compared to ACR4-GFP with DMSO and curcumin treated wild type Columbia. (p< 0.001). (Scale bar = 25µm for A-D).

**Figure 6. ACR4-GFP accumulates in csn5 mutants**

An ACR4-GFP transgene was crossed into a csn5a-1; csn5b-1 double null mutant line and segregating progeny were screened for cop/det/fus phenotype.

(A, B) ACR4-GFP; csn5a-1; csn5b-1 root.

(C, D) ACR4-GFP wild type root.

(E, F) csn5a-1; csn5b-1 root

(G, H) Col wild type root.

(A, C, E, G) Confocal fluorescence images.

(B, D, F, H) Bright field images. Scale bars are 25µm.
Table 1. CSN5A and CSN5B interactions with different cytoplasmic domains of ACR4

<table>
<thead>
<tr>
<th>Sample</th>
<th>BD-KD -LTH SD</th>
<th>β-Gal Activity</th>
<th>BD-CTD -LTH SD</th>
<th>β-Gal Activity</th>
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<tr>
<td>CSN5B</td>
<td>-</td>
<td>-</td>
<td>+</td>
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</tr>
<tr>
<td>CSN5A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

BD-KD: Yeast-two hybrid screen using kinase domain; BD-CTD: Yeast-two hybrid screen using carboxyl terminal domain; -LTH SD: synthetic dextrose (SD) minimal medium with 5mM 3AT and lacking Leu, Trp, His; β-Gal Activity: colony-lift β-galactosidase filter assay for lacZ expression.
Table 2. Primers used for PCR

<table>
<thead>
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<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Strand</th>
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<tr>
<td>CSN5B-3</td>
<td>GACGCTCGACTAGATCCTCTCACAGAATGAGCCTTTGTTCCTCCGATGTAATCATGGGCTCT</td>
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<tr>
<td>CSN5A-5</td>
<td>GCAGGAATTCATTCTAACAGTAGAAGCTCTCAG</td>
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<tr>
<td>CSN5A-3</td>
<td>GACGCTCGACTAGATCCTCTCACAGAATGAGCCTTTGTTCCTCATATGTAATCATAGGGCTCTG</td>
<td>Reverse</td>
</tr>
<tr>
<td>BD-KD-5</td>
<td>CTGGAGATTCGATGAGGATGTAGGT</td>
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</tr>
<tr>
<td>BD-KD-3</td>
<td>AGCCCTCGACATTGTGATGCTATCTCTTC</td>
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<td>BD-CT-5</td>
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<tr>
<td>ACR4FRETB</td>
<td>AGCAGGTACAAATTATGTGCAAGAAAC</td>
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</table>
**Figure 2**

Kinase 1 LRNRCSENDTR.............SS...KDSAEFTK
COOH 1 GSKRSGSENTEFRGGSWITFPSVTSSQRKESA...

kinase 22 DNADEPQLDELCKRRRARVFTYEELEKAADCFKEE
COOH 34 SECDGAEDEGKQEQALRSLEEIGPAFGQSLF

**Figure 3**
Figure 5
CHAPTER 3. CE1LBP1, A NOVEL PROTEIN BINDS THE CE1L ELEMENT OF THE MAIZE VP1 PROMOTER

Antony Chettoor\textsuperscript{1, 3} and Philip W. Becraft\textsuperscript{1, 2, 3}

ABSTRACT

The plant hormone abscisic acid and the transcriptional activator VIVIPAROUS1 (VP1) regulate the transcription of overlapping gene sets during embryo development. Significant progress has been made in elucidating the mechanisms by which VP1 activates transcription, including via the ABA-responsive element (ABRE). However, regulation of \textit{Vp1} is not well understood. Sequence analysis of the \textit{Vp1} promoter identified an ACGT containing ABRE and an element resembling a CE1 element (CE1L). CE1LBP1, a novel protein that binds the CE1L element of the \textit{Vp1} promoter, was isolated in a yeast one-hybrid screen. CE1LBP1 homologs are present in all angiosperms examined, and appear to be a single copy in maize. Using a zinc binding colorimetric assay, we demonstrate that CE1LBP1 binds zinc. Electrophoretic mobility shift assays (EMSAs) show that recombinant CE1LBP1 protein specifically binds to the CE1L element. Antibodies specific to CE1LBP1

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identified CE1LBP1 as part of a DNA/protein complex in supershift EMSAs using nuclear proteins extracted from maize embryos. Identification of the maize CE1LBP1 as a possible trans-acting factor of the *Vp1* CE1L element provides a new link in our understanding of transcriptional regulation during seed maturation and dormancy.

**INTRODUCTION**

Seed development is a crucial process in the life cycle of higher plants, providing the link between two distinct sporophytic generations. According to several plant models, this process can be divided into two stages, morphogenesis and maturation (Gutierrez *et al.* 2007, Weber *et al.* 2005, West and Harada 1993). The morphogenesis phase involves the establishment of an embryo and endosperm by the process of double fertilization. The maturation phase is marked by the arrest of embryo growth, the accumulation of storage compounds, the onset of desiccation tolerance in embryo tissues, finally leading to seed quiescence and usually dormancy. In maize and cereal grains, aleurone, the outermost layer of the endosperm also undergoes the maturation process and remains viable in the dry seed. Seed dormancy is an adaptive trait that promotes the survival of plants in hostile environments (Bewley 1997). In important cereal crops like wheat, dormancy affects yield quality by preventing pre-harvest sprouting (Gubler *et al.* 2005).

Seed dormancy and germination are known to be regulated by several hormones; ethylene, gibberellin, nitrous oxide and brassinosteroids promote seed germination, while abscisic acid seed (ABA) mediates seed dormancy (Finch-Savage and Leubner-Metzger 2006, Finkelstein *et al.* 2002, Koornneef *et al.* 2002, Nambara and Marion-Poll 2003). In
addition, ABA mediates plant responses to various environmental stresses, such as drought, salt, and cold stress (Ingram and Bartels 1996). ABA induces the expression of many maturation-associated genes, such as Em and the late embryogenesis abundant (Lea) genes (Busk and Pages 1998, Marcotte et al. 1989). ABA deficient or insensitive mutants fail to undergo quiescence and precociously germinate, accompanied by decreased expression of maturation genes (Bewley 1997, Koornneef et al. 2002). Of the several ABA-insensitive loci identified to date, three ABI3, ABI4 and ABI5 encode B3, AP2 and bZIP-type transcription factors respectively (Finkelstein and Lynch 2000, Finkelstein et al. 1998, Giraudat et al. 1992, Lopez-Molina and Chua 2000, Lopez-Molina 2000).

Abscisic acid response mutants have been useful to dissect ABA signal transduction pathways (Kucera 2005). The viviparous1 (vp1) and ABA-insensitive (abi3) genes are required for the ABA response associated with late embryogenesis of maize (McCarty, et al. 1989, McCarty et al. 1991) and Arabidopsis (Giraudat et al. 1992), respectively. Maize vp1 mutant kernels are ABA insensitive, undergo precocious germination on the ear, fail to withstand desiccation, and lack anthocyanin pigmentation. (McCarty et al.1989; McCarty et al. 1991). Functional complementation of abi3 mutants by Vp1, and sequence similarities suggests that Vp1 and Abi3 are functionally orthologous genes (Giraudat et al. 1992, Suzuki et al. 2001). VIVIPAROUS1 (VP1) is a B3 domain-containing transcription factor that is central to the regulation of seed maturation in maize (McCarty et al. 1991). VP1/ABI3 homologues are present in most seed plant species (Bobb et al. 1995, Carrari et al. 2001, Hattori et al. 1995, Hollung et al. 1997, Shiota et al. 1998). This indicates that the regulation through VP1/ABI3 is well conserved and is a general theme in the plant kingdom.

Promoters of the barley genes HVA22 and HVA1 contain two types of the coupling elements, CE1 or CE3, that are necessary for activation by ABA (Shen and Ho 1995, Shen et al. 1996). The ABREs and CEs together form ABA-responsive complexes (ABRC1 and ABRC3). The CE1 (ABRC1) and CE3 (ABRC3) are different in sequence and position relative to the ABRE, but both elements are rich in cytosines and guanines (Shen et al. 2004). Biochemical approaches revealed that ABI3 binds to the RY/Sph motif, ABI4 to CE1-like element and ABI5 to ACGT-containing ABRE element (Carles and Koornneef 2002, Kim et al. 2002, Kim 2002, Monke et al. 2004, Monke and H.; Conrad 2004, Niu, Helentjaris and Bate 2002). Furthermore, ABI3 and ABI5, or their rice orthologs, VP1 and TRAB1, have been shown to physically interact (Hobo et al. 1999b, Nakamura 2001). ABI3 and ABI5 bind ABRE elements in response to ABA to regulate gene expression in the maturating seed.

Although considerable information is available on the functions of the Vp1 and ABI3 genes, less is understood about their regulation. Several genes like LEAFY COTYLEDON1 (LEC1), LEC2 and FUSCA3 that regulate the transcription of the Arabidopsis ABI3 gene are known, but the molecular mechanisms by which this regulation occurs are unknown (Kagaya et al. 2005, To et al. 2006). Maize Vp1 expression is induced by ABA and osmoticum during
the mid-maturation embryogenesis phase (Bobb et al., 1995; Hoecker et al., 1995, Cao et al., 2007). A 958 bp (base pair) promoter region of maize VpI was sufficient for the reproduction of the endogenous VpI expression pattern when fused to β-glucoronicidase in transgenic maize. The VpI promoter contains ABRE and CE1-like (CE1L) elements that bind to embryo nuclear proteins (Cao et al. 2007), which represent potential transcription factors that regulate seed maturation. In this study, we describe the identification of CE1LBP1, a novel protein that binds the CE1L element of the VpI promoter. We demonstrate the in-vitro and ex-planta interaction of CE1LBP1 with CE1L element through gel shift and super-shift assays.

**RESULTS**

**Isolation and analysis of CE1LBP1**

To better understand the transcriptional regulation of the VpI and identify upstream candidates in the seed maturation and dormancy process, we performed a yeast one-hybrid screen to isolate cDNA clones that encode proteins, which bind to CE1L (Figure 2). The double-reporter yeast strain containing three 28 bp repeats of CE1L upstream of the His3 or lacZ reporters, failed to grow on selective medium. A cDNA library was constructed from 7 to 20 DAP (days after pollination) embryos using lambda HybriZapII (Stratagene) which fused the cDNA fragments to the yeast GAL4 activation domain in the pGAD424 vector. The reporter yeast strain was transformed with the cDNA library and plated on selective medium. Approximately 3.7 x 10^6 yeast transformants were screened and selected colonies were subsequently re-grown and assayed for β-galactosidase activity using an X-gal filter
assay. 16 clones grew on His selection and showed increased lacZ activity. The isolated cDNA inserts were sequenced and analyzed by BLAST (Altschul and Lipman, 1990). One clone, designated CE1LBpPar grew on His selection and was Lac Z positive (Figure. 3A). It was a partial clone of the sequence GI:85540419 (designated as CE1LBp1 in this study) which encodes a 279 amino acid protein (Figure 3B). CE1LBp1 maps to the chromosome 4L (bin 9) (MaizeGDB, http://www.maizegdb.org). The encoded protein is predicted to be nuclear localized (Hoglund et al. 2006) and does not contain any known protein domains.

**Phylogenetic relationships of the CE1LBp1related proteins**

A BLAST search of NCBI databases identified CE1LBp1 homologs in Rice, Sorghum and Arabidopsis (Altschul et.al., 1990). To address the relationships among the CE1LBp1 related proteins, a multiple sequence alignment was conducted using ClustalW (Figure 4A). Protein sequence alignment of the orthologs showed that Sorghum CE1LBp1 had the highest conservation to CE1LBp1 with 73% amino acid identity, followed by Rice CE1LBp1(japonica) at 40% amino acid identity, Rice CE1LBp1(indica) at 22% amino acid identity and Arabidopsis CE1LBp1 at 18% amino acid identity. The Phylogenetic relationships of the CE1LBp1 proteins are shown in Figure 4B. Importantly, these analyses indicate that CE1LBp1 appears to be a single copy gene in maize and the other species analyzed.

**CE1LBp1 binds to CEIL element in EMSA gel shift assays**

CE1LBp1 was isolated from the yeast- one hybrid screen by its ability to bind the CEIL element of the VP1 promoter. To test whether CE1LBp1 binds the CEIL element in vitro, recombinant CE1LBp1 was used in an EMSA. The full length CE1LBp1 was
expressed as a GST fusion and purified from E. coli RosettaTM cells (Invitrogen). When the 28bp CE1L oligonucleotide was used as a probe, a major retarded band was observed using CE1LBP1-GST protein, indicating that CE1LBP1 bound to CE1L element (Figure 5). The unlabeled CE1L oligonucleotide competed away the retarded band, indicating that the binding was specific (Figure 5). No retarded band was observed using GST alone (Figure 5). This confirms that CE1LBP1 can bind the CE1L element of the Vp1 promoter in vitro.

**Transiently expressed CE1LBP1-GFP is nuclear localized**

To study the in vivo subcellular distribution of CE1LBP1, we expressed a translational fusion between green fluorescent protein (GFP) and CE1LBP1 in onion epidermal cells (Figure 6). As shown in Figure 6C, the CE1LBP1-GFP fusion protein was detected exclusively in the nucleus, indicating that it is targeted to the nucleus. As expected, GFP alone did not localize to a specific compartment and was detected in the cytoplasm and nucleus (Figure 6A).

**CE1LBP1 binds zinc**

Sequence analysis of CE1LBP1 did not identify any known protein domains. The primary amino acid sequence of CE1LBP1 contains a number of cysteines and histidines suggestive of a zinc finger protein, but does not appear to be a canonical zinc finger protein. CE1LBP1 could therefore be a noncanonical zinc finger protein or other type of zinc binding protein. To determine whether CE1LBP1 binds zinc, a colorimetric zinc binding assay using 4-(2-pyridylazo)resorcinol (PAR) was performed on recombinant protein (Hunt et al., 1984; Modrof et al., 2003). When an EDTA treatment followed the Zinc incubation of GST or CE1LPB1-GST, only trace amounts of zinc ions were detected (Figure 7). However when
Zinc incubation followed the EDTA treatment, 3.3 ±0.7 mol of Zn$^{2+}$ per mol of CE1LB1-GST was bound (mean ± sd). In the case of CE1LB1par-GST 0.55±0.06 mol of Zn$^{2+}$ per mol of CE1LB1-GST was bound (average± sd). For GST, only trace amounts of bound zinc was detected.

**Nuclear CE1LB1 binds CE1L**

A previous study performed using 20 DAP embryo nuclear protein extracts identified a specific binding activity to the 28-bp CE1L oligonucleotide probe (Cao et al. 2007). To test the presence of CE1LB1 in the nuclear proteins forming the specific complex on the CE1L region of Vp1 promoter, a supershift EMSA was performed. The two independent mice polyclonal anti-CE1LB1 antisera (pN and pR) were raised and both recognized recombinant CE1LB1 and embryo nuclear proteins specifically whereas the control serum did not (data not shown). Nuclear protein extracts from 20 DAP embryos were incubated with the labeled CE1L oligonucleotide in the presence of anti-CE1LB1 antibodies (pN and pR). As previously reported, nuclear extracts interacted specifically with the probe. Decreased mobility of the complex was observed in the presence of the anti-CE1LB1 antibodies, but not in the presence of control antibodies from pre-immune serum (Figure 8). This incidates that the antibodies recognized CE1LB1 in the nuclear proteins binding to the CE1L oligonucleotide. To confirm the specificity of the interaction of the embryo nuclear proteins to the 28-bp CE1L oligonucleotide, EMSA was repeated and the major retarded band outcompeted by unlabeled CE1L competitor. These results indicate that CE1LB1 present in nuclear protein extracts from maturation stage embryos specifically bound the CE1L probe.
DISCUSSION

VP1 along with ABA and other factors promote seed maturation and dormancy (McCarty 1995). Post transcriptional and posttranslational regulation has been reported for VP1/ABI3 (Bassel et al. 2006, Brady 2003, Zhang et al. 2005). However, transcriptional regulation of VP1/ABI3 has not been extensively studied. Vp1 promoter analysis defined a potential ABRC, consisting of an ACGT core ABRE and a CE1-like motif (Cao et al. 2007). EMSA confirmed that the ABRE and CE1L specifically bound embryo nuclear proteins. In this study, we identify and characterize CE1LBP1, a novel maize DNA-binding factor that binds to the CE1L cis- element of the Vp1 promoter. CE1LBP1 was indentified using the CE1L element of Vp1 in a one-hybrid screen of a 7-20 DAP embryo library (Figure 2).

It is unclear whether the CE1L element of the Vp1 promoter in fact functions as a coupling element in ABA regulated transcription. The only transfactor known to bind CE1 elements is ZmABI4 (Niu et al. 2002). ZmABI4, like the Arabidopsis homolog ABI4 is an APETALA2 (AP2)-like transcription factor that regulates ABA and sugar response genes (Finkelstein et al. 1998). The CE1 element found in sugar-regulated genes, which binds ZmABI4, and the archetypical CE1of Hva22 share a six nucleotide motif (CCACCG), however CACCG appear to be critical for ZmABI4 binding. The CE1L element of Vp1 shares only the CCACC motif with both CE1of Hva22 and the CE1 elements that bind ZmABI4. The distance between the ABRE and CE1L of Vp1 is 50bp. Distances in multiples of 10bp between CE1 and ABREs appear to be critical for ABRC function, but 50 bp is greater than has been previously reported. Furthermore, mutagenesis of core CE1L residues previously reported to be essential for binding or function of CE1 elements did not abrogate
binding (Cao et al., 2007). Taken together, it is possible that the ABRC present in the Vp1 promoter represents a different transacting factor binding site.

The CE1L element of Vp1 and CE1LB1P1 have many of the hallmarks of a trans factor binding site and a trans factor respectively. Gel shift assays using recombinant CE1LB1P1 showed specific binding to CE1L. A CE1LB1P1-GFP fusion was targeted to the nucleus (Figure 6), consistent with its function as a DNA-binding protein. Supershift experiments using anti-CE1LB1P1 polyclonal antibodies demonstrated that CE1LB1P1 was present in embryo nuclear protein extracts and could bind a CE1L oligonucleotide. Overall, these experiments indicate that CE1LB1P1 is a nuclear protein that is capable of binding to the CE1L element of Vp1 in a sequence-specific manner.

CE1LB1P1 is devoid of any known functional protein domains. A striking characteristic of the amino acid sequence is the presence of a number of cysteines and histidines (Figure 3). Although CE1LB1P1 is not predicted to be a canonical zinc finger protein; the sequence suggested the possibility that CE1LB1P1 could bind Zn$^{++}$, which was demonstrated with a colorimetric Zn$^{++}$ binding assay (Figure 7).

A Zn$^{++}$ cofactor is associated with several known transcription factors. p53 is a widely distributed tumor suppressor protein that acts as a sequence-specific transcriptional regulator in response to DNA damage (Prives and Hall 1999, Vousden and Lu 2002). Proper conformation of the p53 DNA binding domain is necessary for DNA binding and transactivation of target genes. Zn$^{++}$ is coordinated to C176 and H179 of the L2 loop and to C238 and C242 of the L3 loops of the protein. Zn$^{++}$ coordination appears necessary for transcriptional activation and removal of Zn$^{++}$ reduces the DNA-binding specificity (Hainaut and Milner 1993, Meplan et al. 2000, Pavletich et al. 1993, Verhaegh et al. 1998).
Interestingly, when p53 and CE1LBP1 sequences are compared, the four residues critical to Zn\(^{++}\) coordination are completely conserved (data not shown). This suggests the possibility that CE1LBP1 could similarly be a Zn\(^{++}\) binding transcription factor.

The developmental expression pattern of CE1LBP1 is as yet unknown. However, based on transcriptome metadata, the putative Arabidopsis ortholog (At 3g63040; Figure 4) is specifically expressed in the maturation phase of seed development. Similarly, the rice ortholog is annotated as embryo-specific. In addition, the expression profile of At3g63040 overlaps with At2g30470 (ABI3) (Nakabayashi et al. 2005), except that ABI3 expression is also seen in vegetative tissues (Winter et al. 2007). Thus it is likely that maize CE1LBP1 expression is also seed specific, consistent with a potential function controlling Vp1 expression during seed maturation. It is also relevant that ZmABI4, which binds CE1, is also specifically expressed during the maturation phase of maize seed development (Niu et al. 2002).

ABI3/VP1 expression has been examined in various species to establish whether it is correlated with seed dormancy. A study utilizing various wild oat (Avena fatua) germplasm with varying degrees of embryo dormancy revealed an increased abundance of AfABI3 transcripts in cultivars with increased embryo dormancy (Jones et al. 1997). However, a similar study using Arabidopsis mutants with various degrees of dormancy did not reveal a correlation between the abundance of AtABI3 transcripts and dormancy (Baumbusch et al. 2004). The fact that dormancy is a complex trait whose control involves strong interactions with many genes and the environment, makes utilizing Quantitative Trait Locus (QTL) analysis a tool of choice. QTL analysis in wheat (Osa et al. 2003) and sorghum (Carrari et al. 2001) did not identify Vp1 as a locus regulating this trait. A major component of the
observed genetic variation for pre-harvest sprouting in wheat appears to be the level of seed dormancy. Therefore identifying QTLs associated with pre-harvest sprouting could provide a better understanding of seed maturation and dormancy. Two QTLs for seed dormancy in wheat, designated as QPhs.ocs-3A.1 and QPhs.ocs-3A.2, were identified on the short and long arms on chromosome 3A, respectively. More of the variation for high dormancy associated with chromosome 3A is ascribable to QPhs.ocs-1 on the short arm than to the QTL on the long arm (Osa et al. 2003). The closest wheat ortholog of CE1LBP1, represented as an EST BJ293947, maps to the short arm of chromosome 3A in addition to 3B and 3D chromosomes. Furthermore, data from the wheat 61k Affimex Microarray suggests elevated expression around 22 DAP, coinciding with seed maturation and seed dormancy. Further studies will address the enticing possibility that CE1LBP1 might play an important role in the transcriptional regulation of VP1 and other genes associated with seed maturation or dormancy in maize and other species.

MATERIALS AND METHODS

Plant materials

The embryos used in electrophoretic mobility shift assay (EMSA) assays and supershifts were collected from B73 or W22 grown in the field, frozen in liquid N\textsubscript{2} and stored in a -50°C freezer.

Yeast one-hybrid screen

The 28 bp oligonucleotide containing the CE1L cis-element from the Vp1 promoter was synthesized (Cao et al. 2007), attaching EcoRI and XbaI adaptors at its 5′ and 3′ ends.
Three tandem repeats of the oligonucleotide were ligated together and cloned into the corresponding restriction enzyme sites of the pHISi and pUC19 plasmids. With EcoRI and SalI restriction sites, the insert containing three tandem copies from pUC19 was cloned into the pLacZi plasmid. The described pLacZi and pHISi plasmid constructs were sequentially transformed into the yeast strain YM4271 to obtain the double-reporter yeast strain. A cDNA library was constructed in the lambda HybriZapII vector (Stratagene) from 7 to 20 DAP embryos, creating fusions of the cDNA fragments to the yeast GAL4 activation domain in the pGAD424 vector. The cDNAs were transformed into the dual-reporter yeast strain and the transformants selected on –HIS selection media. Transformants that grow in the absence of histidine and in the presence of 40 mM 3-AT were selected and tested for activation of the lacZ reporter. The colony- lift filter assay was carried out according to standard protocol (Breeden and Nasmyth 1985).

**Sequence and phylogenetic analysis**

Positive yeast 1-hybrid clones were sequenced and used in BLAST searches (Altschul *et al.* 1990) and the predicted subcellular localizations of the encoded proteins determined by MultiLoc/TargetLoc (Hoglund *et al.* 2006). The sequences of the CE1LBP1 homologs were obtained from NCBI (Maize: GI195607718, Oryza sativa (japonica): GI 115486027, Oryza sativa (indica): GI 85699976, Sorghum: Sb05g022640 and Arabidopsis: At3g63040). 

MegAlign from DNASTAR (Madison, WI) was used to calculate percent identity between CE1LBP1, sorghum CE1LBP1, rice CE1LBP1’s (japonica and indica) and ArabidopsisCE1LBP1. Sequence alignments were carried out using ClustalW2 and MEGA4.
was used to develop phylogenetic relationship using the neighbor-joining method (Kumar et al. 2008).

**Constructions of CE1LBP1-GFP and CE1LBP1-GST fusions**

For construction of the 35S:: CE1LBP1-GFP reporter plasmid, the CE1LBP1 cDNA was amplified by polymerase chain reaction (PCR) with the 5’ primer CE1LPB1GFPF 5’-ACGTTCCTAGATGAGGCGGTGCCGC and the 3’ primer CE1LPB1GFPB 5’-TGACGGATCCACCCACCTCAAGGATGG. The CE1LBP1 cDNA PCR fragment was digested with *Xba*I and *Bam*HI and cloned into the pJ4GFP-XB (Igarashi et al. 2001). For construction of the full length CE1LBP1-GST plasmid, the CE1LBP1 cDNA was amplified by polymerase chain reaction (PCR) with the 5’ primer CE1LPB1GSTF 5’-TAGCGAATTCATGAGGCGGTGCCGC and the 3’ primer CE1LPB1GSTB 5’-GTCACTCGAGTCAACCACCTCAAGGATGG. The CE1LBP1 cDNA PCR fragment was digested with *Eco*R1 and *Xho*I and cloned into the pGEX-5X-1. Both constructs were sequenced and confirmed to be free of mutations.

**Transient expression and visualization of CE1LBP1-GFP in onion epidermal cells**

Onion (*Allium cepa*) transformation was performed essentially as described previously (Varagona et al. 1992). Inner epidermal peels of white onions were placed inside-up on half-strength MS. Onion peels were bomarded using the PDS-1000/He system (DuPont) at 1100 p.s.i. with DNA-coated 1µm gold particles (Bio-Rad). The epidermal tissues were incubated for 20hrs at room temperature prior to analysis. Onion epidermal tissues were mounted in water and viewed under a Nikon inverted confocal microscope. GFP
was visualized using an excitation wavelength of 488 nm and a bandpass 510- to 525-nm emission filter. Under the conditions used, only small amounts of chlorophyll autofluorescence were visualized in untransformed plant tissue.

**Expression and purification of recombinant CE1LBP1-GST protein**

The recombinant full length CE1LBP1-GST plasmids were transformed into *E. coli* RosettaTM cells (Invitrogen). 1mM IPTG was used to induce the expression of these GST-fusion proteins and the proteins were purified from the cell lysate using Glutathione Sepharose™ 4B according to the manufacturers protocol (Amersham Bioscience). The fusion proteins were eluted from the Glutathione Sepharose beads and stored at -80 °C.

**Generation of polyclonal anti-CE1LBP1 antibody**

Mouse anti-CE1LBP1 sera was raised against recombinant CE1LBP1 at the Hybridoma Facility of Iowa State University. Briefly, BALB/c female adult mice were injected subcutaneously with 50 µg of purified recombinant protein. Fifteen days later, the mice were boosted with the same inoculum and 1 month after the initial inoculation, a third boost was administered by intraperitoneally injecting ~50 µg of CE1LBP1 protein/mouse. Fifteen days after this final boost, mice were exanimated by bleeding out, and serum was stored at −80°C. Serum prior to the initial injection of recombinant CE1LBP1 served as the control serum.

*Electrophoretic mobility shift assay (EMSA) and EMSA Supershift assays*

The nuclear protein extracts were prepared from 20 DAP embryos by first using a glycerol-based method for isolation of nuclei (Dorweiler et al. 2000) followed by isolation of nuclear
proteins in protein isolation buffer (10mM Tis-HCl, 1 mM DTT, 0.4 M NaCl (pH 7.5) (Escobar et al. 2001). DNA probes were generated by filling in the 3´ end overhangs of complementary oligonucleotides with ³²P-dCTP (PerkinElmer) and the Klenow fragment of DNA polymerase I (Promega). Labeled probes were subsequently purified using a QIAquick nucleotide removal kit (Qiagen). Binding reactions (25 µL) containing 0.4 picomole of radiolabeled probe, 50 ng of sonicated pBlueScript II SK (Stratagene) and recombinant proteins were incubated in 1 X binding buffer (10 mM Tris-HCl pH 7.4, 50 mM NaCl, 2 mM EDTA, 2.5 mM DTT, 1.25 µg BSA, 0.05 % v/v NP-40, 10 % glycerol) at room temperature for 30 min. Electrophoresis was conducted on a non-denaturing 4.5 % polyacrylamide gel in 0.5 x TBE (45 mM Tris-borate, 0.5 mM EDTA pH 8.0) buffer with constant 70 V for 4 hr at 4 °C. The gels were vacuum dried for 2 hr at 70 °C, and autoradiographed. For the competition assays, unlabeled competitors were incubated with recombinant proteins in 1 X binding buffer on ice for 10 min. After adding the radioactive probe, the binding reactions were further incubated for 30 min at room temperature before electrophoresis was performed, as described. For supershift reactions, 1 µl of CE1LB1 polyclonal mouse antiserum was added 30 min after addition of the radiolabeled probe with an additional 30 min of incubation at 4°C.

**CE1LB1 Zn⁺⁺ binding assay**

The colorimetric Assay for Zn⁺⁺ binding using 4-(2-pyridylazo) resorcinol (PAR) is a modified protocol first developed by Hunt and coworkers (Hunt et al. 1984, Modrof et al. 2003). Briefly, CE1LB1-GST protein complexes were washed with HSD (50mM HEPES-KOH[ph 7.5], 200mM NaCl, 1mM dithiothreitol). Sequential treatment of the fusion protein
complexes with 1mM EDTA and 0.1mM Zinc acetate is carried out. As a control, a similar
treatment of the fusion protein with 0.1mM Zinc acetate and followed by 1mM EDTA is
carried out. Proteins are then digested using Proteinase K prior to the addition of 0.2mM 4-
(2- pyridylazo) resorcinol (PAR). The absorption at 490 nm was measured in a microplate
reader (Dynatech Laboratories, Chantilly, Va.). Zinc ions bound to the fusion protein was
determined by comparison with a Zinc acetate standard curve.

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FIGURE LEGENDS

Figure 1. The predicted features of the 958bp Vp1 promoter. The Vp1, OsVP1 and SbVp1 promoter sequences were subjected to ConSite analysis and analyzed by a PLACE database search to identify cis-acting regulatory elements. The conserved regions between Vp1 and OsVP1 are gray shaded and labeled as PE1 to PE4 and ABRE. 28bp CEIL region is underlined and shown in bold. The predicted binding sites by PLACE search are in bold italics. The DNA sequences used in EMSA are underlined.

Figure 2. Isolation of CE1LBPIcDNA in a yeast one-hybrid assay. Target–reporter constructs containing three copies of the CE1L element regulating the expression of HIS or lacZ were integrated into the YM4271 yeast genome to generate strain CEIL reporter. A cDNA library from 7-20 DAP embryos was ligated into a yeast expression vector to generate a library of plasmids encoding embryo cDNA–GAL4 activation domain fusion proteins. This library was transformed into CEIL reporter. Clones encoding putative DNA-binding cDNAs were then isolated by their lacZ activity and their ability to grow on selective synthetic drop-out (SD) medium (SD –Leu/–His + 30mM 3-AT). The clone encoding partial CE1BP1 demonstrated strong lacZ expression in filter lift assays using X-Gal (visible as blue staining), while CEIL reporter showed no background lacZ expression. The control strain
used in the screen lacked the CEIL element inserted upstream of HIS3 or lacZ reporter genes. YPAD (complete media); -HIS (selective media) and LacZ (β-galactosidase assay).

**Figure 3.** CE1LBp1 and CELBPpar sequences. cDNA (EL01N0323B09) was used to predict the CEILBP1 ORF. (3A) Nucleotide and (3B) deduced amino acid sequence of the CEILBP1 ORF. CELPpar, the partial clone isolated from the yeast-one hybrid screen is shown in bold.

**Figure 4.** Comparison and phylogeny of CE1LBp1 with close homologs. (A) Alignment of deduced amino acid sequence of maize CE1LBp1: GI195607718, *Oryza sativa* (japonica): GI 115486027, *Oryza sativa* (indica): GI 85699976, sorghum: Sb05g022640 and Arabidopsis: At3g63040. Single amino acid residues were aligned, and the consensus sequence is presented underneath as asterisks. (B). Phylogeny was inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Phylogenetic analyses were conducted in MEGA4

**Figure 5.** CE1LBp1 binds specifically to the CEIL element in EMSA. Each DNA-protein binding reaction contained 0.4 picomole $^{32}$P labeled CEIL with or without 1 µg recombinant CEILBP1 protein. CEIL bound to CEILBP1 recombinant protein causing one major retarded band. The retarded band could be competed away with 100X unlabeled CEIL. Recombinant CELPB1 was expressed as GST fusion protein and used in the gel retardation assay. Open arrow: retarded band; closed arrow: free probe.
**Figure 6.** CE1LBP1 is a nuclear localized protein. The intracellular localization of CE1LBP1 was investigated by transient assay of a GFP fusion protein in onion epidermal cells. (A,B) As control, GFP alone was expressed in onion cells. GFP localizes to the cytoplasm and nucleus. (C,D) CE1LBP1–GFP was mainly localized to the nucleus. Confocal fluorescent images (A and C) and bright field images (B and D) of the same cells. Arrows indicate positions of onion epidermal cell nuclei. The bar in (B) represents 50 µm for all images.

**Figure 7.** CE1LBP1 binds zinc. Binding of zinc ions by GST and CE1LBP1 was measured by a colorimetric assay. CE1LBP1-GST and CELBPpar-GST were immobilized on GST beads and treated with EDTA (1mM) followed by zinc acetate (0.1mM) or vice versa. The eluted CE1LBP1-GST and CELBPpar–GST was digested with proteinase K and treated with 4-(2-pyridylazo) resorcinol (PAR). GST was used as a control. PAR forms a complex with Zn²⁺ that was monitored by an increased absorption at 490nm. The average and standard deviation of 5 replicates are reported.

**Figure 8.** CE1LPB1 nuclear protein binds specifically to the CE1L element of Vp1. 20 DAP embryo nuclear proteins bound to CE1L causing a retarded band, which could be competed away by 100X unlabeled CE1L. Supershift assays were performed with embryo nuclear protein extracts and polyclonal mouse antiserum raised to CE1LBP1 (N and R). The supershifted bands indicate the antibodies recognized the CE1LBP1 protein in a complex with the CEIL oligonucleotide. Preimmune serum (PI) was used as a negative control. Dark lined arrow: supershifted band; open arrow: retarded band; closed arrow: free probe.
-891 GTCGACACC CATATTAGAAGACGCCAGTCATCTTTTTTTGTAATGCACAG
-841 GTGGGAAAAATGCTAAGAAGACATAGTACATAGAGCCAAAGATGAAAT
-791 GAGGAGGACCGCAGATATAAATAAATAAAAAAGCCCGAGGAGTTTGCAAAAGGAA
-741 CTCCTACTACTAGTACAATCCACCCATCTAAGTATACCTACACGCAAAGTT
-691 CCCAAATAGAAGGTTCCACTCTCACTTCACTTCACTTCGCGGCCCTTCAAGTCC
   PE4
-641 CCGAATCACC CACCACCTGTTAGGTTCCGCAGTCGTAGAGAGAACTCC
-591 TCTGCTTTCGTAGACAAGCCAGTGGCACCATCCTGGAGCTTCCGGTGG
-541 GATCACACGCAGAGACTGCTAGTGATGGAATCTGGATCCACACACTCGGG
   PE3
-491 ATTCA GTCGGC GCGCCGCGAGACACCTCATCTACGCGGCGCAGACAGCT
-441 ACGGCACGCTGTTTTTCCTCACTACGACCGGCTCGGAGGCCACGCCTCAT
-391 TGACTGACC AAAAAAACATGGGCCGACTCTCTTCCTGACTGAGCAGCTC
-341 AAATTAGACGCGGCCAAGGCGAGACCTGAAAAACACACAGACACTGAC
-291 ATGTGGACAGCAGCGCTCGGCCCATGCCATAGACGGTCATGCACAGAG
-241 AAACCTAATCAGATAATAGTTTGTGATACCTCTATGACAAGATGGGCTCATT
-191 TATCTTCAGCCCCACGTCTCGGAGAGAGGTATAGGGTGCGCGCCGGGCTGGG
   PE2
-141 GAGAAGTATGGTGCCGAGGAGAGGCTGCCGAGTCGGAGAGCGCTCC
   ABRE/
-91 CTCGGTCGCCACGTTGTGCACGCAGCCCGCATCTCGGTCTCCCTCCTCTCTC
   CE1L
-41 TCTCCTCTTTTCTCCCTCTCCACCTCTCTGCTTCACGTCTCTCA
   PE1
+10 GTAACGGCACCCACCTCCACACCCCTACCGAGAAGGGCCCGCGCCCGCGACAGAC
+60 ACAGACAC
YM4271

CEIL Reporter

Vector control

CEILBP1

Figure 2
**3A**

ATGAGGCGGTGCCGCCCGCGCGGACTGCCGACATGGAGCAAGAGTAGGTACGGTGCAGGG
CAGCGAGAGCGCGGGCAAGTGCCGGGAGAGGTCGAGCGCCACCACCGCGGGACTGGCCGGTGGGAGAGGCAGCGCTGCCTGATGGACTGCAAGAGCCGGGAGCGGGAGGAGGAGGGCGGAGCGGCGAGGCAGCGGGCAACGACAGCGACGCGGCGCGACGCCGAGGAGGACAGCGGCGACCGCTGCCCTTGCGAGAAGCAGTGCGAGGGCCACCACGACCGGGAAAGCAGGCAGCAGTGCGTCCAAGCTTGCAGCGCCGGCGACAGCAGCGCGGCGGCAGCCGTGACTCCAACGTCGACGAGGACGACTGACGACCGGGCGCTGCCAGAAGATGCCAGCACCACAGCGATCGGCGAGGCAGTGGTGCGTGCAGCGCTGCGAGCGTAAGCAACAGGAGGAGGACGCCGCCCGCGACGA

**3B**

MRRCRRRLAVDMEQEGYGRGSESAGKCRERCRERHHRGDWWERQRLMDCKSREREEDGGGS<br SGGRGEEDSGDRCPEKQECEGHDRESRQCVQACERRRRQRRGGRSRDNVDDEEDSRDRCQMKCRRRHRQARQWCVQRCRKQEEDAADDNSGRCQKRCQHHSQDRQARQWC

**Figure 3**
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Figure 8
CHAPTER 4. GENERAL CONCLUSIONS

CONCLUSIONS

Genetic studies suggest that a hierarchical system regulates the acquisition of aleurone cell fate and subsequent differentiation of aleurone. In this thesis, I reported two studies, which will help to understand endosperm cell fate specification and differentiation; one involved in early and the other in late differentiation. CR4 is critical for early aleurone cell fate specification in maize and active forms of the Arabidopsis CR4 ortholog, ACR4, are rapidly turned over. We present data that suggests the CSN5 subunit of the COP9 signalosome (CSN) is involved in the rapid turnover of ACR4. Vp1 is the most upstream known transcriptional regulator in the control of anthocyanin production in the aleurone. In the second part of this thesis, I report the identification and characterization of CE1LBP1, a putative transcription factor that binds to the CE1-like element of the Vp1 promoter.

The cr4 gene encodes a receptor-like kinase with a tumor necrosis factor receptor (TNFR)-like motif in the extracellular domain. Disruption of aleurone cell fate in cr4 mutants suggests that CR4 could function in the perception and transduction of the positional cues that specify aleurone cell fate (Becraft et al. 1996). In this thesis, I describe the characterization of proteins that interact with ACR4 and might regulate the ACR4 signal transduction pathway. A yeast two-hybrid screen with the ACR4 cytoplasmic domain resulted in the identification of CSN5A and CSN5B, two Arabidopsis CSN5 subunits of the CSN complex. The interactions were confirmed with in-vitro pull downs and ACR4 was capable of phosphorylating the CSN5 proteins. In-vivo FRET experiments confirmed that
ACR4 was in close proximity to CSN5A and CSN5B within the context of a plant cell. The interactions with CSN5 subunits suggested the CSN might be involved in ACR4 protein turnover. Treatment of plants with curcumin, a CSN inhibitor, resulted in the accumulation of ACR4-GFP in roots. ACR4-GFP also accumulated in csn5a-1 csn5b-1 double mutants, confirming the role of CSN5 in ACR4 turnover.

It is been more than a decade since the identification of CR4 in Maize. However, compared to other RLK’s in plants, progress in characterization of CR4 signal components and transduction has been limited. The lack of detectable levels of receptor protein has been the limitation in the utilization of biochemical, molecular and cell biology approaches (Gifford et al. 2005). The work presented in this thesis is significant in identification of CSN as a factor involved in ACR4 turnover. The major function of the CSN is ascribed to regulating ubiquitination of protein substrates to trigger the protein degradation pathway. This study for the first time reports that a RLK is in contact with the CSN subunit (CSN5). The interaction between a CSN subunit has not been demonstrated in well characterized RLK’s like BRI1 or FLS2 (Kinoshita et al. 2005, Robatzek et al. 2006). I hypothesize that this interaction between the CSN and ACR4 could be responsible for the undetectable levels of ACR4 receptor protein, whereas BRI1 and FLS2, which apparently don’t interact with the CSN, are readily detectable.

In this study, I show that curcumin, a CSN complex inhibitor that has been used extensively in animal systems, can also be used in plants (Chaudhary and Hruska 2003, Uhle et al. 2003). The use of the inhibitor results in accumulation of ACR4 presumably by inhibiting turnover. The slowing down of receptor turnover could provide an opportunity to biochemically characterize interactors that bind to the receptor. Identification of the ligands
for the receptor is critical to understanding the signal transduction pathway initiated at the membrane and transduced into the cell resulting in cellular responses. Based on the extracellular domain of ACR4, the ligand is proposed to be proteinacious in nature. In the case of mammalian EGF/EGFR receptor interaction, EGF is bound to the EGFR at the membrane, internalized and finally degraded (Hanover et al. 1984). It is possible that the putative ligand of ACR4 induces receptor internalization and degradation. The use of curcumin could possibly slow down receptor turnover and thereby provide an opportunity to detect the ligand bound to the receptor. Experiment design would involve treating seedlings with curcumin, followed by protein crosslinking, pull down of receptor-ligand complexes and detection of the ligand using mass-spectroscopy.

To define the role of CSN5 subunit interactions with ACR4, it is required to map the binding sites on the cytoplasmic domain of ACR4. Two processes that could modulate the receptor signaling include phosphorylation and ubiquitination. ACR4 being a serine/threonine kinase, phosphorylation occurs on serine and threonine residues, while ubiquitination occurs on lysine residues. In-vitro and in-vivo interaction assays of mutant versions of ACR4 with CSN5 would provide an insight into amino acid residues critical to the interaction. The results from these experiments would guide in-planta experiments to address the functional implication of ACR4 turnover in the context of aleurone cell fate. I hypothesize that stable trangenics expressing ACR4 versions with mutations in key cytoplasmic residues could impair receptor turnover. Furthermore, introduction of the cognate versions of CR4 into maize could address the functional role of CR4 receptor turnover in aleurone cell fate specification. The completion of these proposed experiments on
ACR4 / CR4 receptor turnover would advance our knowledge of this important class of plant RLKs.

In the second study, I focus on VP1, which is expressed in the embryo and aleurone, and plays an important regulatory role in late embryogenesis and seed maturation in *Zea mays* (McCarty *et al.* 1989). It also regulates the expression of *C1*, a transcription factor regulating anthocyanin genes in aleurone cells (Suzuki *et al.* 1997). In many plant species, the inception of dormancy, which is a part of the seed maturation process, is mediated by abscisic acid (ABA) and VP1 (Giraudat *et al.* 1992, McCarty *et al.* 1991). Significant progress has been made in elucidating the mechanism of ABA and VP1 regulated gene expression, including the characterization of an ABA-responsive element (ABRE) (Busk and Pages 1997, Hobo *et al.* 1999a, Shen *et al.* 1996). However, the mechanism of *Vp1* regulation has not been well understood. In this thesis, I describe a trans factor that interacts with a CE1-like cis element of the *Vp1* promoter. CE1LBP1 was isolated from a one-hybrid screen. Structure-function predictions of CE1LBP1 did not identify any known protein domains; however CE1LBP1 was confirmed to bind zinc using a colorimetric zinc binding assay. CE1LBP1-GFP fusions localize to the nucleus, consistent with a role of CE1LBP1 as a transcriptional factor. Electrophoretic mobility shift assays with recombinant CE1LBP1 and a CE1-like oligonucleotide confirmed the binding specificity of CE1LBP1 to the CE1-like element. Super shift assays with embryo nuclear extract and antibodies raised against recombinant CE1LBP1 demonstrated that CE1LBP1 is part of an embryo DNA/protein complex and therefore could function as a trans factor that regulates *Vp1* expression via the CE1-like cis element of the promoter.
The identification of CEILBP1 as putative CE1L transacting factor is significant for a number of reasons. (1) CEILBP1 is only the second known CE1L transfactor other than ZmABI4. (2) CEILBP1 has no domains of known function, unlike the APETALA2 (AP2) domain present in ZmABI4. (3) Dormancy levels in small grains correlate with Vp1 expression but QTL’s do not map to Vp1 locus suggesting that factors that regulate Vp1 expression could also regulate dormancy levels. Thus, it is possible that CELPB1 could regulate seed dormancy.

A putative ABRC1 was identified in the Vp1 promoter (Cao et al. 2007), however several items raise the question of whether the CE1-like element actually functions as a coupling element. Although the spacing between the ABRE and CE1L elements fit the “multiple of 10 rule”, 50 nucleotides is further than the 20 nucleotides found in the well characterized ABRC1 in HVA22 (Shen et al. 2004, (Shen and Ho 1995). In addition, mutagenesis of the CE1L element did not abrogate binding of nuclear proteins as would be expected from published studies (Cao et al. 2007, Shen et al. 2004, Niu et al. 2002). Thus, this element in the Vp1 promoter might not function as a coupling element and could have a function unrelated to ABA responses. In the present study, I focused on identifying CEILBP1 as a nuclear protein that binds the CE1L element of the Vp1 promoter during embryo maturation. I propose using RT-PCR to characterize the developmental profile of CEILBP1 expression through the maturation process. Characterization of the CEILBP1 binding site on the Vp1 promoter has not yet been completed. To carry out an unbiased consenses DNA-binding motif of CEILBP1, I propose the systematic evolution of ligands by exponential enrichment (SELEX) method. This experiment could define the element and help clarify the nature of CE1-like element present in Vp1 promoter. To address the
functional role of CE1LBP1, databases of publically available mutant collection were scanned. Due to the lack of any available mutants, reverse genetic approaches like RNA interference (RNAi) and/or targeting induced local lesions in genomes (TILLING) will be required to target \( CE1LBP1 \). \( CE1LBP1 \) is predicted to be a single copy gene in maize, which should allow targeting by the above methods.

The one-hybrid screen used in this study used an embryo specific library to identify CE1LBP1. In cereals and maize in particular, the aleurone along with the embryo undergo maturation and dormancy (Bewley 1997). It will be interesting to characterize the role of CE1LBP1 in aleurone cells, and to determine the similarities and differences in \( Vp1 \) regulation between embryos and aleurone cells.

CE1LBP1 can bind \( Zn^{++} \) and does not contain any domain of known function. p53, the tumor suppressor protein, binds \( Zn^{++} \) through a highly conserved quartet of three cysteines and one histidine and functions as a transcription factor (Hainaut and Milner 1993, Pavletich \textit{et al.} 1993, Prives and Hall 1999, Verhaegh \textit{et al.} 1998). CE1LBP1 shares complete conservation of these four residues with p53. It is hypothesized that the conserved amino acid quartet of CE1LBP1 might also be critical in binding \( Zn^{++} \). To test that possibility, \textit{in vitro} \( Zn^{++} \) binding assays of mutant CE1LBP1 proteins that harbor mutations in the four amino acids of the conserved quartet could be carried out.

Taken together, these two studies attempt to fill in some of the gaps in our understanding of aleurone cell fate and differentiation. From the first study, CSN5 of the CSN complex functions in CR4 regulation and from the second, CE1LBP1 can be placed upstream of \( Vp1 \).
REFERENCES


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