# IOS Preliminary Proposal: Collaborative Research - CPSF30 at the convergence of cellular signaling and RNA Processing

## I. Personnel

University of Kentucky

PI – Dr. Arthur G. Hunt, Professor, Dept. of Plant and Soil Sciences. Dr. Hunt will direct the research at the University of Kentucky and will be responsible for directing the research of junior scientists (one graduate student and undergraduates), overall coordination, manuscript writing, and reporting.

Miami University

Co-PI, Dr. Q. Quinn Li, Professor, Botany Department, Miami University. Dr. Li will lead the efforts at Miami University, and will advise a postdoctoral scientist and graduate student, perform data analysis, and assist with manuscript writing and reporting.

## II. Project Description

### A. Conceptual Framework– the importance of mRNA polyadenylation

In recent years, mRNA polyadenylation has been recognized as an important step for the regulation of gene expression [1-7]. This mode of regulation (alternative polyadenylation, or APA), in which one of a number of potential poly(A) sites is chosen in favor of others, can impact the ultimate expression of a gene in a number of ways. Thus, the final mRNA may have altered stability, different translatability, or modified subcellular locations, depending on the composition of the 3'-UTR. The final mRNA may also encode a truncated protein that may itself be either non-functional or possessed of altered functionality. APA can have a significant impact on growth and development. This is perhaps best exemplified by reports that link alternative poly(A) site choice with changes in cell growth and differentiation in mammals [8-11]. APA plays vital roles in plants, affecting aspects of plant growth and physiology as far-ranging as primary amino acid metabolism [12], ethylene signaling [13, 14], RNA processing [15], self-incompatibility [16], and flowering [17-19].

### B. Background and Significance – AtCPSF30 links cellular signaling, alternative polyadenylation, and plant growth (includes supporting data)

While the scope of APA in plants is known to be broad, the mechanisms that lead to changes in poly(A) site choice, and that connect APA with growth and development, are less-well understood. In these contexts, the Arabidopsis 30 kD subunit of the so-called Cleavage and Polyadenylation Specificity Factor (AtCPSF30) is of particular interest. Calmodulin inhibits RNA binding by AtCPSF30 in a calcium-dependent fashion [15]. AtCPSF30 is also inhibited by sulfhydryl reagents [20] that act to disrupt a disulfide linkage in the protein [21]. This suggests that AtCPSF30 may be regulated through oxidation and reduction of the disulfide linkage, as occurs with other regulatory proteins (e.g., [22-28]). This possibility is consistent with reports that a mutant (*oxt6*) deficient in AtCPSF30 exhibits a greater tolerance to oxidative stresses than wild-type plants [29].

In addition to the reported tolerance to oxidative stresses [29], *oxt6* plants are also altered in their responses to ACC (the precursor to ethylene), auxin, cytokinin, and gibberellic acid (M. Liu, A. G. Hunt, Q. Q. Li et al., submitted), and they are deficient in lateral root (Fig. 1B) and flower

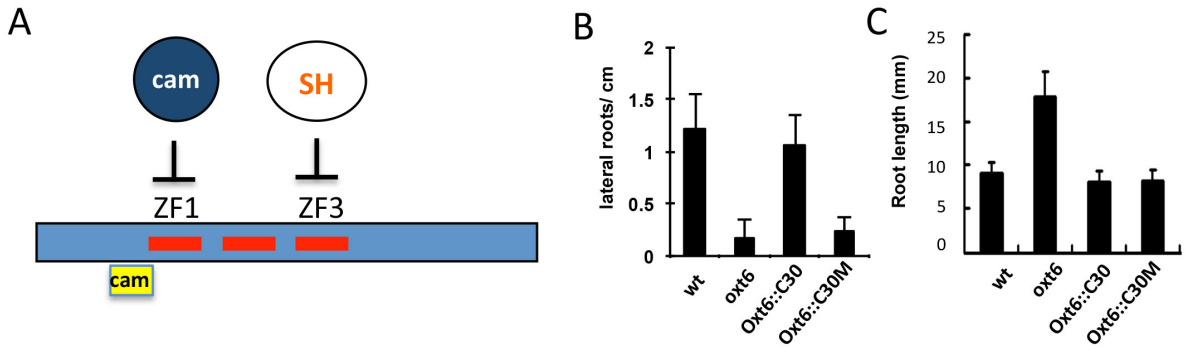


Figure 1. A. Model for the structure of AtCPSF30, derived from published reports [15,20,21,31]. The three zinc finger motifs of the protein are represented as red lines within the blue box that denotes the full 250 amino acid protein. The calmodulin binding site ("cam", yellow box) is shown beneath the blue box. The zinc fingers that are targets of inhibition by calmodulin ("ZF1") and sulfhydryl reagents ("ZF3") are indicated as shown. ("SH" = sulfhydryl reagents.) In panels B and C, and in the text, "C30" represents the wild-type protein, and "C30M" a mutant that does not bind calmodulin. The mutant retains all of the other activities (RNA binding, in particular) of the wild-type protein [15,31]. B. Lateral root numbers of the wild-type, *oxt6*, *oxt6::*C30, and *oxt6::*C30M plants. (In the latter two lines, transgenes encoding the wild-type AtCPSF30 (C30) or calmodulin-binding mutant (C30M) were expressed in the *oxt6* mutant background.) Plants were grown from seed on vertical plates for 12-16 days and lateral root numbers counted. The primary root lengths for the various lines were indistinguishable. C. Responses of the wild-type, *oxt6*, *oxt6::*C30, and *oxt6::*C30M plants to methyl viologen. Assays were performed as described [29]. The root lengths of plants grown in the absence of MV were indistinguishable (not shown).

development (M. Liu et al., submitted). For all of these phenotypes, wild-type growth can be restored by expressing AtCPSF30 (C30 in Fig. 1) in the *oxt6* background ([29]; also, Fig. 1 and M. Liu et al., submitted). However, expression in the *oxt6* background of a form of AtCPSF30 that does not bind calmodulin (C30M in Fig. 1) fails to restore wild-type lateral root growth (Fig. 1B) or responses to auxin, cytokinin, or gibberellic acid (M. Liu et al., submitted); this mutant does restore wild-type flower development as well as responses to oxidative stress (Fig. 1C) and ACC (M. Liu et al., submitted). These results show that AtCPSF30 has both calmodulin -dependent and –independent roles in growth and development.

In terms of mRNA polyadenylation, in the *oxt6* mutant, there is a widespread change in poly(A) site utilization that affects between 45% and 90% of all expressed genes [30]. These changes have the potential to alter mRNA functionality in many ways. Together with the biochemical studies summarized above, this suggests that a consequence of the activation of Ca/calmodulin or redox signaling pathways will be an inhibition of AtCPSF30 activity and concomitant wide-ranging changes in poly(A) site choice.

The many characteristics to which AtCPSF30 has been linked constitute, separately and in combination, vital emergent properties of the plant, and include both developmental processes and the responses of plants to the environment. The proposed research will further an understanding of how multiple signaling cues are often resolved into distinctive molecular outcomes important for growth and development.

## C. Hypotheses/Specific Aims

The model for the role(s) of AtCPSF30 as a multifaceted regulatory hub is illustrated in Figure 2. The specific aims of the research proposed herein address different "levels" of this model (yellow circles in Fig. 2). Stated as hypotheses to be tested, these are:

1. Different signaling inputs are processed, through AtCPSF30, into different sets of APA events and outcomes ("1" in Fig. 2).
2. AtCPSF30-mediated APA alters mRNA functionality of direct RNA targets of the protein ("2" in Fig. 2).
3. AtCPSF30-mediated APA controls the expression of genes involved in lateral root development ("3" in Fig. 2).

## D. Experimental Plan

***Hypothesis 1:*** AtCPSF30 is the target of two potential signaling mechanisms (calmodulin and redox), each of which impacts a different zinc finger motif (Fig. 1A; [15, 20, 21, 31]). This raises the possibility that different signaling inputs affect poly(A) site choice in different sets of genes. To test this, transgenes that encode variants of AtCPSF30 that possess inactivating mutations in the respective zinc finger motifs (ZF1 and ZF3; these mutations are described in [31]) will be expressed in the *oxt6* mutant. These alterations of AtCPSF30 should approximate the consequences of inactivation of AtCPSF30 by calmodulin and redox signaling. The

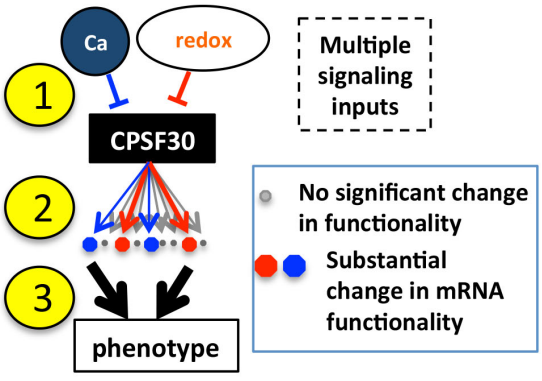


Figure 2. Model for the functioning of AtCPSF30 in signaling, gene regulation, and growth and development. "Ca" = calcium signaling, through calmodulin; "redox" = regulation via oxidation/reduction of the disulfide bond in AtCPSF30. The gray, blue, and red circles and arrows extending beneath the black box that represents AtCPSF30 denote individual genes whose polyadenylation sites are regulated by AtCPSF30; blue symbols represent mRNAs whose functionality changes in response to calcium signaling, and red symbols those that change in response to redox signals. For ease of presentation, hypothetical mRNAs whose function change in response to both calcium AND redox signaling are not illustrated. Numbered yellow circles on the left refer to the specific aims that are described in the text.

resulting lines will be assessed for the phenotypes seen in the *oxt6* mutant (tolerance of oxidative stress, lateral root and flower development, and responses to the growth regulators listed in the preceding subsection). In parallel, genome-wide poly(A) site choice in the *oxt6* mutant will be compared with that in the wild-type and in *oxt6* mutant plants that express different mutant forms of AtCPSF30. Genome-wide poly(A) site choice will be determined as described [30, 32].

The expected outcome of this study is that plants that have different AtCPSF30 genotypes (e.g., *oxt6* plants that express transgenes encoding AtCPSF30 isoforms that have mutations in either ZF1 or ZF3) will be different from each other and from the *oxt6* mutant. This would suggest that inactivation of different zinc finger motifs, as would occur upon activation of calmodulin or redox signaling pathways, leads to a different set of changes in poly(A) site choice, and subsequently to different phenotypic outcomes. Such an outcome would identify CPSF30 as a conduit that receives different signaling inputs and produces a specialized output for each. Alternatively, mutant lines that express different forms of AtCPSF30 may be indistinguishable from each other, and also from the *oxt6* mutant. This outcome would support a model whereby differing signaling inputs converge on AtCPSF30 to yield common outcomes; this may provide a posttranscriptional mechanism for cross-talk between signaling pathways.

***Hypothesis 2:*** The finding that poly(A) site choice in a large majority of Arabidopsis genes is altered in the *oxt6* mutant [30] is interesting and important, but it leaves open the question of how APA contributes to phenotype. One explanation is that a subset of Arabidopsis genes is affected by AtCPSF30-mediated APA such that the encoded mRNAs have altered functionalities. To test this, the translatabilities and stabilities of mRNAs in the wild-type and *oxt6* mutant will be assessed on a global scale.

To study the possibility that CPSF30-mediated APA affects the translatabilities of target mRNAs, the experimental approach described elsewhere [33, 34] will be adopted. A FLAG-tagged form of RPL18 will be expressed in the wild-type and *oxt6* mutant, polysomes will be rapidly immunopurified from extracts prepared from transgenic plants, and the 3' end profiles of the associated mRNA populations determined (as in [32]). Poly(A) sites associated with mRNAs with low translatabilities will be those that are under-represented in polysomal mRNAs, while sites associated with mRNAs with enhanced translatabilies will be those over-represented in polysomes. The wt-*oxt6* comparison will permit the identification of AtCPSF30-dependent sites that affect mRNA translatability.

mRNA stability will be studied using the experimental approach described by Gutierrez et al. [35]. Briefly, plants will be grown on defined agar media, transferred to an incubation buffer, treated with cordycepin (a transcriptional inhibitor), and sampled at various times for RNA isolation. The genome-wide poly(A) profiles in these samples will then be determined. The resulting data will be used to estimate the stabilities of the various mRNA isoforms in the wild-type and *oxt6* mutant; computational approaches similar to those developed for other projects in the PIs' labs [30] will be used for this.

The expected outcome is that a subset of mRNAs will exhibit altered translatabilities and/or stabilities in the *oxt6* mutant. This would link AtCPSF30-dependent APA with definitive molecular consequences. Combined with the results expected in the previous subsection, these data will lead to a functional map of the network of genes that are controlled by AtCPSF30. The alternative outcome, that AtCPSF30-mediated APA has little effect on global mRNA functionality, will necessitate a substantial revision of the models for how AtCPSF30 contributes to gene expression.

***Hypothesis 3:*** In the *oxt6* mutant, the frequency of initiation of new lateral root primordia (stages I and II as defined in Malamy and Benfey [36]) is reduced (data not shown), such that the numbers of lateral roots are much lower in the mutant (Fig. 1B). The wild-type AtCPSF30, but not a calmodulin-insensitive mutant protein, restores normal lateral root growth to the *oxt6* mutant (Fig. 1B). Based on this, it is hypothesized that AtCPSF30 regulates the expression of genes required for lateral root development in ways that require the calmodulin-AtCPSF30 interaction.

This hypothesis will be tested by studying gene expression and poly(A) site usage in xylem-pole pericycle cells, the progenitors of lateral roots. This effort will capitalize on the ability to rapidly isolate and characterize specific root cell types [37, 38]. For this study, an enhancer-trap GFP fusion (JO121) that is specific for root xylem-pole pericycle cells [39] will be used. This reporter will be introduced into the *oxt6*::AtCPSF30 and *oxt6*::AtCPSF30m lines. Xylem-pole pericycle cells will be isolated using cell sorting technology (Dr. Li's lab has been using a facility at Cincinnati Children's Hospital), and RNA isolated from the purified cells. Genome-wide poly(A) site choice and gene expression will be determined using approaches in practice in the PIs' laboratories [29, 30, 32]. From the differences between the *oxt6*::AtCPSF30 and *oxt6*::AtCPSF30m lines, two sets of data will be obtained: differentially expressed genes (DEG) and genes that exhibit APA (APAG). The set of DEG will be genes whose expression change directly as a result of calmodulin-dependent AtCPSF30-mediated APA as well as genes whose expression changes are secondary. The set of APAG will consist of those genes whose poly(A) site choice change as a consequence of calmodulin-dependent signaling through AtCPSF30. The overlap between these gene sets will constitute a set of putative primary targets that are affected by AtCPSF30 in a calmodulin-dependent fashion.

The expected result of this study is that one or more genes known to be important for the initiation of lateral root primordia [40] will prove to be primary targets of calmodulin-dependent AtCPSF30-mediated polyadenylation. This will confirm the stated hypothesis and lend credence to the larger model that holds that various of the direct targets of AtCPSF30 are responsible for the different phenotypes that are seen in the *oxt6* mutant. The alternative outcome, that genes vital for lateral root initiation are not subject to calmodulin-dependent APA, will necessitate a revision of this hypothesis; an attractive possibility would be that the changes in the expression of genes involved lateral root initiation are consequences of APA of mRNAs encoded by "upstream" genes in the hypothetical genetic network.

### E. Broader Impacts

An understanding of how signaling cues act on the polyadenylation complex to yield specific responses will provide new insight into the mechanisms by which abiotic and developmental stimuli affect crop productivity. Logical extensions of the expected findings include predictive and proactive methodologies that would ameliorate the effects of stresses on crop plants.

In addition to incorporation of the results and theory into classes taught by the PIs, the computational expertise and the datasets that will be generated will be made available to an NSF- and HHMI- funded consortium of colleges and universities (DBI 1061893, http://www.hhmi.org/news/hhmicolleges20120524.html) that has the goal of developing undergraduate laboratory and classroom exercises in high through-put DNA sequencing. The consortium includes small liberal arts colleges, state-supported institutions, and Historically Black Colleges and Universities (HBCUs). Dr. Hunt has lectured at one participating institution and will serve as an external reviewer of sub-projects to be administered by the consortium.

Other outreach activities that are current in the PIs' groups include: a collaboration with a member (Dr. Chi Shen) of the Dept. of Computer Science at Kentucky State University (an HCBU) that engages a KSU undergraduate in bioinformatics research; a collaboration with a teacher (Mr. Patrick Thomas, first author on Thomas et al. [30]) at Franklin-Simpson High School in Franklin County, KY (a rural school), also involving computational research; and participation (by Dr. Li) in an NSF-funded Undergraduate Research Mentorship project intended to recruit and mentor minority students in research in cell and molecular biology at Miami Univ.

### F. Comments on the collaboration between the PIs

The PIs were co-PIs on a previously NSF-funded collaborative project (IOS-0817818) that resulted in the publication of three reviews [7, 41, 42] and twelve research papers [21, 30, 32, 43-51]. The two laboratories routinely exchange data and techniques and conduct periodic joint lab meetings (at one of the two locations, and also in conjunction with the annual RustBelt RNA Meeting held at locations in Ohio). The success of this approach to interaction and communication is reflected in numerous jointly-authored publications (e.g., [15, 29, 30, 32, 51]).

## III. Literature cited


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