

Small yet effective

The Ethylene-responsive element binding factor-associated Amphiphilic Repression (EAR) motif

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Abbreviations: ERF, ethylene-responsive element binding factor; EAR motif, ERF-associated Amphiphilic Repression motif; TR, transcriptional regulator; TF, transcription factor; HMM, hidden Markov model; PHI-BLAST, pattern hit initiated-BLAST; DPM, degenerate pattern matching; bHLH, basic helix-loop-helix; G₂, golden2; ARF, auxin response factor

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The Ethylene-responsive element binding factor-associated Amphiphilic Repression (EAR) motif is a small yet distinct regulatory motif that is conserved in many plant transcriptional regulator (TR) proteins associated with diverse biological functions. We have previously established a list of high-confidence Arabidopsis EAR repressors, the EAR repressome, comprising 219 TRs belonging to 21 different TR families. This class of proteins and the sequence context of the EAR motif exhibited a high degree of conservation across evolutionarily diverse plant species. Our comprehensive genome-wide analysis enabled refining EAR motifs as comprising either LxLxL or DLNxxP. Comparing the representation of these sequence signatures in TRs to that of other repressor motifs we show that the EAR motif is the one most frequently represented, detected in 10 to 25% of the TRs from diverse plant species. The mechanisms involved in regulation of EAR motif function and the cellular fates of EAR repressors are currently not well understood. Our earlier analysis had implicated amino acid residues flanking the EAR motifs in regulation of their functionality. Here, we present additional evidence supporting possible regulation of EAR motif function by phosphorylation of integral or adjacent Ser and/or Thr residues. Additionally, we discuss potential novel roles of EAR motifs in plant-pathogen interaction and processes other than transcriptional repression.

Introduction

Transcriptional repression has emerged as an important regulatory mechanism essential for establishing intricate patterns of gene expression during plant development and plant responses to hormonal and stress signals. In the past decade, a few transcriptional repression motifs have been identified in plants, including the EAR motif,¹ TLLLFR motif,² ^R/_KLFGV motif³ and LxLxPP motif.⁴ The EAR motif was initially identified in a subset of class II ERF and C2H2 family proteins as a small motif containing a conserved sequence pattern of ^L/_FDLN^L/_F(x)P which when tethered to transcriptional activators converted them into dominant repressors.¹ Subsequent identification of the EAR motif and confirmation of its repression capability in several other TR proteins involved in a broad range of biological processes⁵⁻¹¹ has unequivocally established a role for this motif in mediating transcriptional repression. Furthermore, recent discoveries of corepressors interacting with the EAR motif, such as TPL¹² and AtSAP18,^{7,13} have begun to unravel the mechanisms of EAR-mediated repression. To gain insight to the potential breadth of utilization of the EAR motif in plant gene regulation, we conducted a comprehensive bioinformatics analysis (employing a HMM/PHI-BLAST based approach) of the Arabidopsis proteome and generated a high-confidence list of EAR repressors, the EAR repressome, comprising 219 TR proteins belonging to 21 different TR families.¹⁴ Based on the sequence comparison of core EAR motif sites in ERFs

Table 1. Active repression motif-containing transcriptional regulators in various plant species

Plant species	Number of annotated transcriptional regulators ^a					
	Total	EAR motifs		LxLxPP	R/ _K LFGV	TLLLFR
		LxLxL	DLNxxP			
Arabidopsis	1770	352	73	40	29	1
Papaya	671	145	14	10	3	0
Poplar	2576	419	72	25	25	0
Grapevine	890	194	25	8	6	0
Rice	2384	469	76	74	35	0
Sorghum	483	103	25	12	5	0
Maize	764	106	2	5	2	0
Sugarcane	1177	129	5	1	0	0
Soybean	1891	277	52	16	10	0
<i>Medicago truncatula</i>	1022	149	29	8	8	0
<i>Lotus japonicus</i>	457	78	6	3	3	0
<i>Physcomitrella patens</i>	1170	193	21	18	14	0

^aNumber of transcriptional regulators containing EAR motif(s) in Arabidopsis is as per Kagale et al.¹⁴ For remaining proteomes, datasets were downloaded on February 09, 2010 from Plaza¹⁹ (papaya, grapevine and sorghum) or PlantTFDB²⁰ (remaining plant species) databases, and were screened by degenerate pattern matching for different transcriptional repression motifs.

and C2H2 family proteins, as well as the novel proteins identified in our study, we refined the consensus sequence patterns of EAR motifs as LxLxL or DLNxxP. In the Arabidopsis EAR repressome, the LxLxL type of EAR motif occurs more (~3-fold) frequently than the DLNxxP type.¹⁴

Addenda to the EAR Repressome

A notable absence in the high-confidence EAR repressome collection was members of the bHLH class, a highly abundant group of TFs in plants. Using DPM analysis, we identified 179 additional TRs containing EAR motif-like sequences.¹⁴ This collection included not only 23 bHLH TFs but also members of the G₂-like (11 proteins), MYB-related (10 proteins), ARF (8 proteins) and 31 other TF families (one to seven proteins each). A potential role in transcriptional repression has recently been revealed for at least one of the EAR motif-containing bHLH proteins. PIF4 (At2g43010), a LxLxL type of EAR motif-containing bHLH protein, is predicted to interact with PIF7 forming a heterodimer implicated in repression of DREB genes under circadian control.¹⁵ In addition, several of the ARF proteins, such as ARF2, 11, 14, 15 and 20–22, are implicated as candidate repressors based on their high structural similarity to ARF1, which is implicated in transcriptional

repression.^{16,17} Of the other TFs identified by DPM analysis, further work will not only ascribe functionality of EAR motifs in these proteins but also highlight the importance of this mechanism of gene regulation in plant biology. Our analysis supports the EAR motif being represented in 39 of the 64 classes of TRs currently annotated in Arabidopsis (PlantTFDB; datf.cbi.pku.edu.cn/), suggesting EAR-mediated gene regulation as being a principle mechanism of gene regulation recruited multiple times during evolution to control plant gene expression.

We have shown that the EAR motif is conserved in orthologues of Arabidopsis EAR repressors across evolutionarily diverse plant species.¹⁴ To further expand the repertoire of the EAR repressome across multiple plant genomes and gain insight on the evolutionary convergence of repressor motifs, we performed a DPM analysis screening for EAR, LxLxPP,⁴ R/_KLFGV³ and TLLLFR² motifs. As shown in Table 1, 10 to 25% of the TRs from various plant species, including papaya, poplar, grapevine, rice, sorghum, maize, sugarcane, soybean, *Medicago truncatula*, *Lotus japonicus* and *Physcomitrella patens* (a moss), contain an EAR motif-like sequence(s). In contrast, the representation of LxLxPP (<3%) and R/_KLFGV (<2%) although also conserved is markedly reduced, and the

TLLLFR motif was found only once (in AtMYBL2). This analysis illustrates that the EAR motif appears to be the most predominant form of transcriptional repression motif so far identified in plants and implies a strong conservation of this mechanism of regulating gene expression.

Regulation of EAR Motif Function

The chemical nature of amino acid residues integral and adjacent to the EAR motif may influence the exposure of the EAR motif to potential corepressors and determine its availability for interaction with or binding affinity for the corepressors; additionally, the sequence context of the EAR motif may affect nuclear localization, repression strength and turnover of EAR repressors. Interestingly, the Ser and Thr residues in the EAR motif regions of at least five proteins in Arabidopsis, namely IAA9, ERF10, BEH4, DEAR4 and a C2H2 family protein, were identified to be phosphorylated,¹⁴ supporting the possibility of regulation of EAR motif function by phosphorylation. Here, we show that the Ser and Thr residues in IAA9, BEH4 and DEAR4 that were determined to be phosphorylated are conserved in corresponding orthologues across evolutionarily diverse plant species, including papaya, poplar, grapevine, rice and

sorghum (Fig. 1), consistent with these Ser and Thr residues possessing functional relevance retained during evolution. Consequently, identification of the protein kinases and phosphatases affecting the phosphorylation status of EAR motif containing proteins, and thereby potentially regulating their function, is essential to understanding possible post-translational regulation of EAR-mediated gene regulation. We have recently identified a protein kinase that interacts with the EAR motif (Kagale S and Rozwadowski K, unpublished) and are currently exploring its potential role in regulating EAR motif function.

Potential Novel Roles of the EAR Motif

A recent discovery of an EAR motif in XopD, a type III effector protein from *Xanthomonas campestris* pathovar *vesicatoria*, and its essentiality for pathogen virulence and effect on host transcription in tomato¹⁸ has opened a new avenue of research exploring the role of the EAR motif in plant-pathogen interactions. Preliminary sequence analysis of several plant pathogenic proteomes indicates that sequences similar to the EAR motif are conserved in several of the effector proteins that may be required for virulence in corresponding pathogens (Kagale S and Rozwadowski K, unpublished). Future studies aimed in this direction will identify a novel role for the EAR motif and advance our understanding of host-pathogen interaction.

Although the EAR motif is currently exclusively implicated in transcriptional repression, our DPM analysis also identified ~5,200 non-transcription related proteins in Arabidopsis containing the EAR motif.¹⁴ This discovery prompts us to speculate a role for this motif in processes other than transcriptional repression, acting perhaps to facilitate protein complex formation in non-transcription related proteins. It is also possible that this motif occurs in these proteins without any functional relevance. The establishment of the biological significance of the EAR motif in non-transcription related proteins is an area that needs to be explored further.

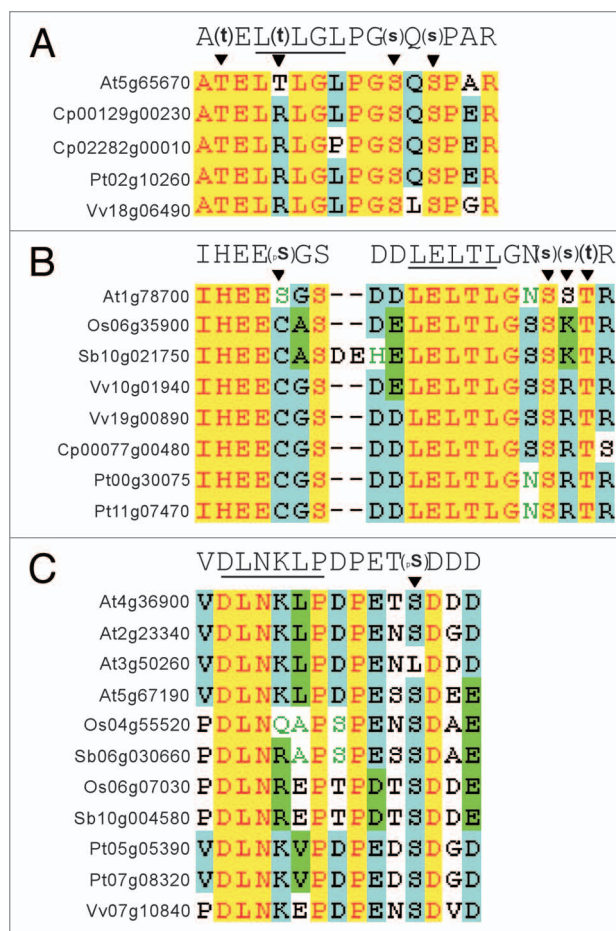


Figure 1. Evolutionary conservation of phosphorylated Ser and Thr residues in EAR motifs of IAA9, BEH4 and DEAR4. The EAR motif and adjacent residues of orthologues and paralogues of Arabidopsis IAA9 (A), BEH4 (B) and DEAR4 (C) from papaya (Cp), poplar (Pt), rice (Os), sorghum (Sb) and grapevine (Vv) are represented. The phosphorylated peptide sequences (shown at the top of each alignment) are annotated using the modification nomenclature outlined in the publication guidelines of Molecular & Cellular Proteomics. An experimentally confirmed phosphorylation site is indicated in boldface letters with a lowercase p and is contained in parentheses: (pS). pST sites wherein which residue(s) within the peptide is phosphorylated but remains ambiguous are presented as boldface lowercase letters, and each potential candidate (not necessarily all S and T residues) is contained in parentheses: (s) or (t). The sequence of the core EAR motif site is underlined.

Future Studies

Research conducted over the past few years has advanced our understanding of the EAR motif and its role in negative regulation of gene expression in plants; however, the role of the EAR motif in ~80% of the Arabidopsis EAR repressome candidates remains unknown. Future biochemical and genetic studies are required to determine the exact functions of the EAR motif in these candidate proteins. Identification of potential interacting partners of the EAR motif, structural characterization of their physical interaction and role of

post-translational modifications are also essential in understanding the mechanism of EAR motif-mediated transcriptional repression. Understanding the regulation of EAR motif functions and the cellular fate of EAR motif containing proteins will not only provide new insight to the link between gene regulation and plant biology but also provide a novel framework for genetic improvements of crops.

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