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Transcriptional regulation of chickpea ferritin *CaFer1* influences its role in iron homeostasis and stress response

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ABSTRACT

Ferritin, ubiquitous among all living organisms except yeast, exhibits iron-regulated expression. In plants, this regulation is applied through transcriptional control. Previous studies established the presence of two types of *cis*-acting elements in the promoter region: the iron regulatory element (FRE) in soybean and the iron-dependent regulatory sequence (IDRS) in maize and *Arabidopsis*. Adverse environmental conditions (e.g. water-deficit and oxidative stress) are known to modulate the expression of phytoferritin genes. In this study, we cloned and investigated the promoter sequence of a chickpea ferritin, designated *CaFer1*. Phylogenetic analysis of the *CaFer1* promoter revealed its evolutionary relationship with other phytoferritins. The *CaFer1* promoter exhibited several putative regulatory elements including two known transcription factor (TF) binding sites, Athb-1 and Myb.Ph. Electrophoretic mobility shift assay confirmed the sequence-specific binding of Athb-1 and Myb.Ph on the *CaFer1* promoter. The TF-binding dynamics of CaFer1 showed high induction under conditions of iron-deficiency and water-deficit. We also demonstrated the possible interaction of CaFer1 with IRT1, a key component of the iron uptake system in plants, indicating its involvement in maintaining cellular iron levels. These results provide new insights into the underlying mechanisms of function of these interacting factors in CaFer1-mediated iron homeostasis and the stress response in plants.

1. Introduction

Iron is essential for numerous cell functions and is particularly associated with cell growth and proliferation (Heath et al., 2013). Nonetheless, excess iron can lead to oxidative stress by inducing the generation of reactive oxygen species via the Fenton reaction (Dixon and Stockwell, 2014). Metal homeostasis, in general, requires efficient regulatory mechanisms to coordinate the uptake and storage of specific metals (Winge et al., 1998). These mechanisms often involve metal sensing proteins that behave as *cis*-acting regulatory factors to influence the enhancement or repression of gene expression (Hentze and Kuhn, 1996). Iron, being the most abundant metal, is tightly regulated to prevent both iron toxicity and deficiency (Guerinot and Yi, 1995). The ubiquitous iron-storage protein, ferritin, plays a key role in this regulation owing to its buffering and transitory storage capacity.

The expression of ferritin, in eukaryotic cells, is regulated by levels of intracellular iron. In plants, iron overload induces transcription of the ferritin gene, whereas it de-represses ferritin mRNA translation in mammalian cells (Petit et al., 2001a). In effect, regulation of phytoferritin occurs at the level of transcription in plants (Lescure et al., 1991) and at the level of translation in animals (Theil, 2007; Arosio et al., 2009). The regulation of phytoferritin has been studied in maize (Lobreaux et al., 1992; Lobreaux et al., 1993; Savino et al., 1997; Petit et al., 2001b), pea (Lobreaux and Briat, 1991) and soybean (Lescure et al., 1991; Wei and Theil, 2000). A 14 base-pair-long iron-dependent regulatory sequence (IDRS) was previously identified in the promoter of *ZmFer1* and *AtFer1* (Petit et al., 2001b), which is putatively involved in the suppression of their synthesis under conditions of iron-deficiency (Petit et al., 2001b; Tarantino et al., 2003). Unlike *ZmFer1*, the regulation of *AtFer1* in response to iron and H_2O_2 occurs via two different pathways (Gaymard et al., 1996; Arnaud et al., 2006). The expression of ferritin is also regulated through nitric oxide, which acts via IDRS downstream of iron (Murgia et al., 2002). It has been suggested that IDRS-binding transcription factors (TFs) are required but are not adequate for *AtFer1* de-repression (Arnaud et al., 2006). Even though both age- and dark-induced senescence lead to the activation of the *AtFer1* promoter, IDRS is involved only in facilitating dark stress activation (Tarantino et al., 2003; Murgia et al., 2007). Interestingly, the regulation of *AtFer1* expression by phosphate starvation does not involve IDRS and acts independently of iron (Bournier et al., 2013). Taken together,

Abbreviations: Fe, iron; EMSA, electrophoretic mobility shift assay; IDRS, iron-dependent regulatory sequence; FRE, Fe responsive element; IRE, iron-responsive element; ABA, Abscisic acid; ABRE, ABA binding element; PHR1, phosphate starvation response1

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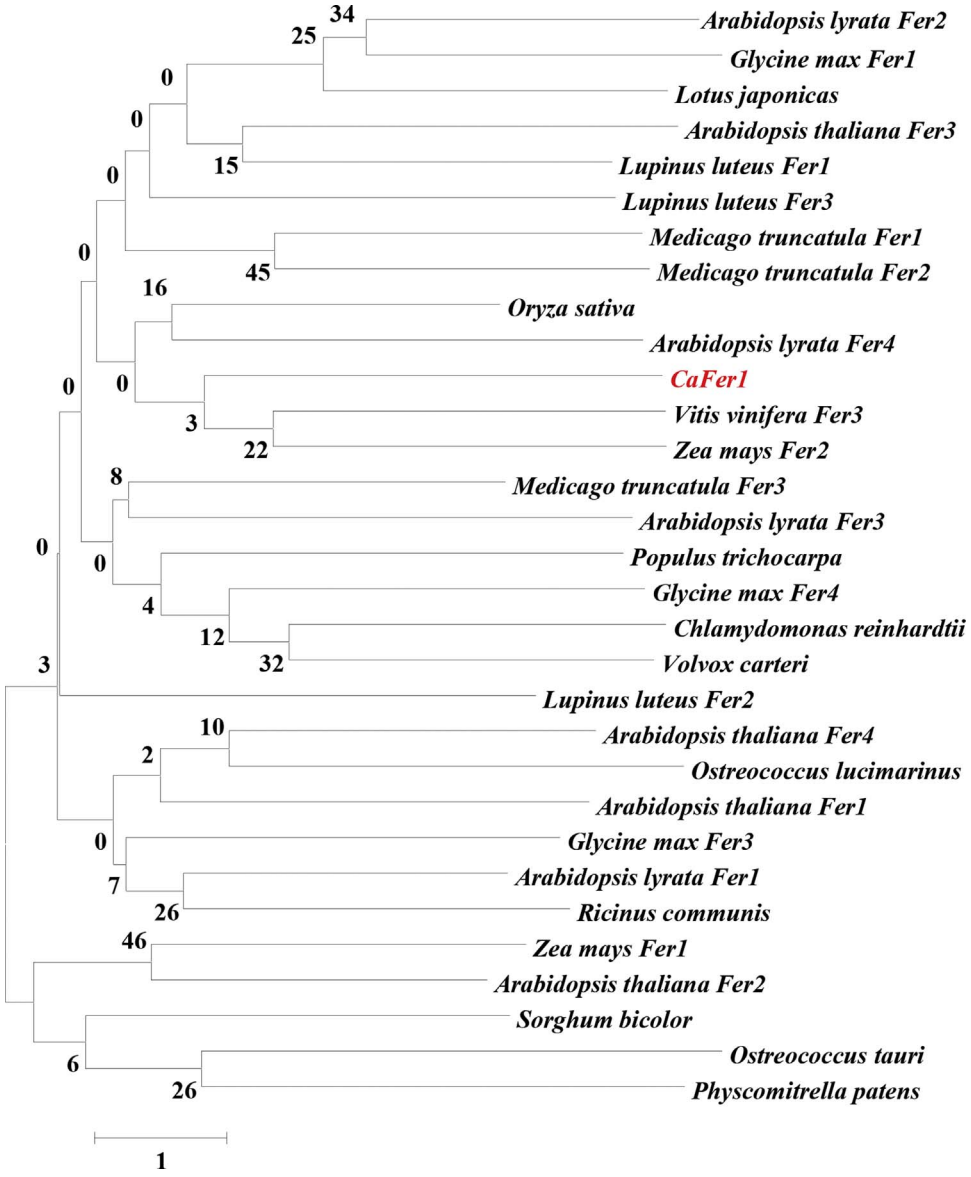

Fig. 1. Phylogenetic analysis of the *CaFer1* promoter. The evolutionary relationship of the *CaFer1* promoter with that of other phytoferritins was analyzed by phylogram using MEGA v 5.0. The bootstrap value was set at 1000; a neighbor-joining method was used to construct the phylogram. Branches were drawn to scale with the bar indicating 1 substitution per site. Different clades of phytoferritins from various plant species are shown in the phylogram.

it is apparent that we are still far from a complete understanding of the pathways involved in the regulation of phytoferritin. The fact that there is so much diversification in the regulation and function of ferritin across the plant kingdom adds to this confusion. While ferritins are not considered to be the prime source of iron for the function of chloroplasts in *Arabidopsis* (Ravet et al., 2009), a ferritin deficiency causes rapid degradation of photosystem I in *Chlamydomonas* (Busch et al., 2008; Long et al., 2008). The role of phytoferritin in iron storage varies across species, accounting for 98% of stored iron in legumes, but only 5% in *Arabidopsis* (Marentes and Grusak, 1998; Ravet et al., 2009). Indeed, pathways involved in regulating the expression of phytoferritin are poorly understood. Furthermore, the inducers and/or repressors involved in the pathways and their crosstalk are still not known. To complicate matters further, members of the ferritin superfamily within the same species are also regulated in distinct and diverse ways, adding complexity to the regulation network.

Most cellular proteins function as part of a complex with other proteins and regulate each other's functions. Therefore, one important way to characterize the biological role/s of a protein is to identify its interacting partners. Co-immunoprecipitation is one of the most common methods for verifying the interaction of two proteins (Berggard et al., 2007). Pull-down/co-immunoprecipitation is widely used to identify interacting associates of a target protein, including those that interact only transiently, yet it never confirms that the interacting proteins are truly and physically linked. It is possible that two co-immunoprecipitating proteins are linked together by a third protein that acts as a scaffold (Hall, 2005). Therefore, it is ideal to combine co-immunoprecipitation experiments with techniques that utilize purified proteins, such as fusion protein pull-downs. In an earlier study, we identified a stress-responsive ferritin, designated *CaFer1*, from chickpea and partially characterized it, suggesting a synergistic role of CaFer1 in stress tolerance and iron homeostasis (Parveen et al., 2016). The current study was aimed at enhancing our understanding of *CaFer1* regulation, especially in response to conditions of iron availability and water deficiency, through the identification of interacting partners. We assessed the putative *cis*-acting and *trans*-acting regulatory elements, and characterized the binding of two putative TFs, Athb-1 and Myb.Ph, on the promoter. Furthermore, we used multivariate bioinformatics tools and co-immunoprecipitation techniques for the comprehensive identification of CaFer1-complex components.