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Arabidopsis ILITHYIA protein is necessary for proper chloroplast biogenesis and root development independent of eIF2α phosphorylation

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ABSTRACT

One of the main mechanisms blocking translation after stress situations is mediated by phosphorylation of the α-subunit of the eukaryotic initiation factor 2 (eIF2), performed in Arabidopsis by the protein kinase GCN2 which interacts and is activated by ILITHYIA(ILA). ILA is involved in plant immunity and its mutant lines present phenotypes not shared by the *gcn2* mutants. The functional link between these two genes remains elusive in plants. In this study, we show that, although both ILA and GCN2 genes are necessary to mediate eIF2α phosphorylation upon treatments with the aromatic amino acid biosynthesis inhibitor glyphosate, their mutants develop distinct root and chloroplast phenotypes. Electron microscopy experiments reveal that *ila* mutants, but not *gcn2,* are affected in chloroplast biogenesis, explaining the macroscopic phenotype previously observed for these mutants. *ila3* mutants present a complex transcriptional reprogramming affecting defense responses, photosynthesis and protein folding, among others. Double mutant analyses suggest that ILA has a distinct function which is independent of GCN2 and eIF2α phosphorylation. These results suggest that these two genes may have common but also distinct functions in Arabidopsis.

1. Introduction

Translational arrest of existing mRNAs is a quicker way to control gene expression than transcriptional regulation and allows adaptation to sudden appearance of stresses. However, cells cannot survive very long if protein synthesis is arrested. Therefore, the process of translational arrest has to be tightly regulated to assure cell survival, so that it remains active only until the cell has overcome the immediate impact of the stress (Roy and von Arnim, 2013).

In animals and yeast, one of the main mechanisms to inhibit translation after stress situations is the one mediated by the phosphorylation of the α-subunit of the eIF2 translational initiation factor. This factor is responsible for binding of the initiator methionyl-tRNA^{Met} and delivering it to the 40S ribosome. When the initiator codon is found, eIF2-GDP is released, and the protein is elongated. The exchange of GDP for GTP, catalyzed by the eIF2B factor, is needed for new rounds of translation (Hinnebusch, 2005). Phosphorylation of the eIF2α factor under stress situations provides then a rapid way for translational arrest, as phosphorylated eIF2α is a competitive inhibitor of the less abundant eIF2B. This process is transient, and specific phosphatases dephoshorylate again eIF2α once the cell has initiated cellular responses to cope with the stress situation (Rojas et al., 2014). These responses include the translation of specific mRNAs, as together with the global translational inhibition, eIF2α phosphorylation leads to preferential translation of specific mRNAs. This is the case, for instance, of the GCN4 gene in yeast, a transcription factor that is translated during the general protein synthesis arrest that follows eIF2α phosphorylation after stress situations, and that will subsequently activate a battery of genes involved in the recovery for the stress (Hinnebusch, 2005).

In vertebrates, four different kinases are known to phosphorylate eIF2α (Hinnebusch, 2005). Plants, however, equally to *Saccharomyces cerevisiae*, have only one of these kinases, named GCN2, and different stresses has been shown to activate eIF2α in a GCN2-dependent manner

*Abbreviations:*GCN, General Control Non-derepresible

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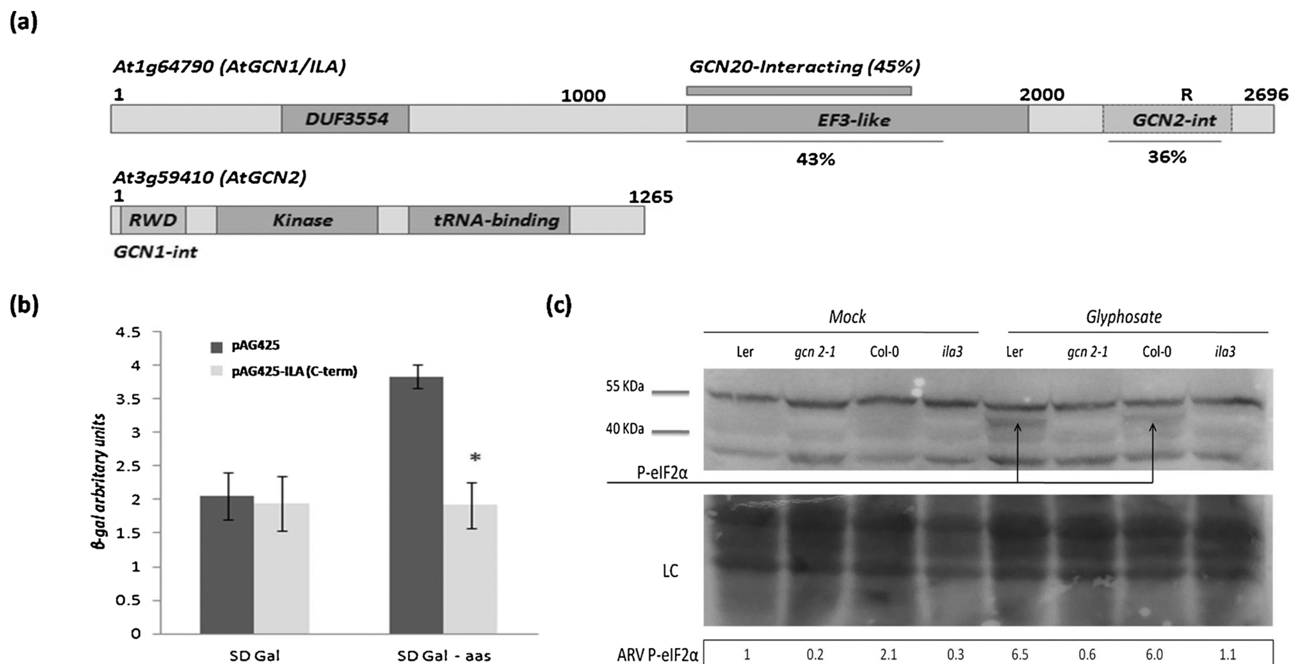

Fig. 1. Interaction of ILA with GCN2. (a). Protein structure of At1g64790 ILITHYIA (ILA) and At3g59410 (AtGCN2), showing conserved domains of both proteins. Amino acid positions are indicated. Identity (BLASP) with the corresponding yeast (*Saccharomyces cerevisiae*) GCN1 protein is shown in percentage for ILA. DUF3554: domain of unknown function predicted by InterPro; EF3-like, GCN20-interacting, GCN2-int: predicted domains according to homology to *S. cerevisiae* GCN1 protein. Conserved arginine residue in the GCN2-interacting domain of ILA is highlighted. RWD (Nameki et al., 2004): Region of interaction with GCN1, termed after three major RWD-containing proteins: RING finger-containing proteins, WD-repeat-containing proteins, and yeast DEAD (DEXD)-like helicases; GCN1-int: GCN1-interacting domain in the GCN2 protein according to Nameki et al., 2004; Kinase: Conserved Kinase domain in the GCN2 protein, t-RNA binding: t-RNA binding domain in the GCN2 protein (Zhang et al., 2003). (b) β-galactosidase assays (arbitrary units) over yeast strains harboring the p180 reporter, transformed with the empty pAG425 vector, and pAG425 containing the C-terminal end of the ILA gene (pAG425-ILA(C-term)). Cells were grown in SD medium with galactose (SD gal), and SD gal without amino acids (SDgal − aas). * indicates significant differences after *t*-test (p-value < 0.001) between pAG425 and pAG425-ILA(C-term). Three independent experiments were performed. **(c)**. Western blot assaying eIF2α phosphorylation on wild-type (Col-0), *ila3*, wild-type (Ler) and *gcn2-1* seedlings, mock-treated and treated with glyphosate to induce eIF2α phosphorylation. A differential band corresponding to P-eIF2α is shown by arrows. Equal amount of protein (20μg) was loaded in a 10% SDS-PAGE gel. LC: Loading control. Adjusted Density Values (ADV) for the samples was calculated by dividing the relative density of its sample lane by the relative density of the loading control for the same lane.

(Lageix et al., 2008; Zhang et al., 2008). Initially characterized in yeast as a kinase activated under amino acid starvation, reports are constantly emerging on new biological aspects where GCN2 is involved, being activated by a considerable number of stress situations different from amino acid starvation (reviewed in Castilho et al., 2014).

The current model for GCN2 activation proposes that upon amino acid starvation, accumulated uncharged tRNAs bind to a regulatory domain in GCN2 that resembles histidyl-tRNA synthetase (HisRS-related), inducing a conformational change in the protein that exposes the kinase domain for activation. Activation of GCN2 further requires binding to GCN1, which forms a complex with the ATP-binding cassette protein GCN20, both attached to ribosomes. The N–terminal domain of GCN2 contains the region needed for interaction with GCN1 (Sattlegger and Hinnebusch, 2000).

The existence of a GCN2 gene in Arabidopsis (AtGCN2) suggests that a GCN-dependent pathway for eIF2α phosphorylation is also conserved in plants. Some of the abovementioned aspects of GCN2 function seem to be present in Arabidopsis. Besides the kinase domain, the AtGCN2 protein includes the conserved N-terminal GCN1-interacting and the HisRS-related domains, and it has been proved to interact with uncharged tRNAs and to have activity on eIF2α isoforms of Arabidopsis (Li et al., 2013). Moreover, the Arabidopsis gene complements the yeast *gcn2* mutant (Zhang et al., 2003), and an Arabidopsis *gcn2* knock-out mutant line is unable to phosphorylate eIF2α (Zhang et al., 2008). However, although it seems clear that AtGCN2 phosphorylates eIF2α under many different stresses, whether this process activates translational arrest in a similar way to mammals and yeast is controversial and the lack of total understanding persists (Immanuel et al., 2012).

One of the aspects that remained undetermined was the existence of a GCN1 protein in plants and its role on GCN2 activation and eIF2α phosphorylation. In yeast, GCN1 is absolutely required for GCN2 to detect uncharged tRNAS, and, as a result, *gcn1* knock-out strains are unable to activate GCN2 and phosphorylate eIF2α under amino acid starvation (Marton et al., 1993). GCN1 is a protein containing HEAT repeats (from huntingtin, elongation factor 3, phosphatase 2A and TOR1, proteins that also contains these domains, proposed to serve as interaction sites for other proteins), and homology to the eEF3 elongation factor exclusive to fungus, required for the ATP-dependent release of deacylated tRNA from the ribosomal E-site during protein biosynthesis in these organisms. The very C-terminal region is determinant for GCN2 interaction (Sattlegger and Hinnebusch, 2000).

In Arabidopsis, ILITHYIA (ILA) is the only protein in the genome presenting similarity to GCN1 (57% similarity over the C-terminal most conserved region). Initially identified as a protein necessary for embryogenesis (Johnston et al., 2007), it has been implicated in plant immunity against bacterial infections. In particular, the ILA protein was shown to be required for basal and non-host resistance against *Pseudomonas syringae*, as well as resistance conditioned by specific resistance (R) genes, effector-triggered immunity (ETI) and systemic acquired resistance (SAR) (Monaghan and Li, 2010). ILA is also a long protein (2696 amino acids) that contains HEAT repeats in their middle region. Phenotypes of *ila* mutants include yellow leaves with aberrant shape and male sterility, indicating a pleiotropic role in plant development (Monaghan and Li, 2010). Very recently, the involvement of ILA in mediating the phosphorylation of eIF2α through GCN2 activation has been reported (Wang et al., 2016).

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