Proteomic analysis of the *ila2* mutant of *Arabidopsis* links translational regulation with photosynthesis, protein folding and ribosomal proteins

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Abstract

A key component of the conserved GCN (General Control Non-repressive) signalling pathway in plants is the ILITHYIA (ILA) protein, homologous to the yeast GCN1 protein. Similar to yeast and animals, ILA seems to be involved in the activation of the eIF2 α kinase GCN2, allowing protein arrest under stress conditions. Recently, it has been reported that, in plants, ILA could be also playing a role in development independent of GCN2. To gain insight into this new function of the ILA protein, we have performed proteomic analysis to identify differentially-expressed proteins in the strong loss-of-function allele of ILA (*ila2*). *ila2* plants present a reduced expression of photosynthetic proteins, and an increased expression of the translational initiation factor eIF5A, ribosomal proteins and heat-shock proteins. These results open new hypothesis to understand the roles of this important translational regulator in plant tissues.

Keywords Proteomics · Translational regulation · Protein folding · GCN1 · Photosynthesis

Introduction

Eukaryotic cells employ complex mechanisms to regulate protein synthesis at multiple levels. Control of translational initiation, that requires only hours to take effect, provides cells with a mechanism to rapidly induce or repress synthesis of specific proteins. This is especially important for plants, which must quickly respond to changing environmental conditions.

A major signalling pathway for stress responsive translational control is the one mediated by the conserved protein kinase GCN2 (for General Control Non-repressive). This signalling pathway enables yeast cells to sense and overcome amino acid deprivation (Hinnebusch 2005). Upon activation of this kinase, the α subunit of the initiation factor eIF2 is phosphorylated and inhibited because it stays bound to the guanine nucleotide exchange factor eIF2B. As eIF2 is essential for loading a tRNA onto the 40S ribosome, overall translation initiation is arrested, while allowing the selective translation of the transcription factor GCN4. A GCN2 kinase exists in all plant species studied so far, and eIF2 α is phosphorylated by many situations in a GCN2-dependent manner, including amino-acid deprivation stresses such as glyphosate treatment (Zhang et al. 2008; Faus et al. 2015). However, recent data by Izquierdo et al. (2018) argue against P-eIF2 α involvement in the general abolition of protein synthesis. Besides, the absence of a GCN4-like transcription factor in the *Arabidopsis* genome or the lack of susceptibility of *gcn2* mutants to this and other abiotic stresses support the idea of a minor relevance of GCN2 and P-eIF2 α regulation in plants under stress.

A key component of this signalling pathway is the protein GCN1, necessary in yeast and animals for eIF2 α phosphorylation. GCN1 promotes GCN2 activation by

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uncharged t-RNAs by positioning GCN2 on the ribosome (García-Barrio et al. 2000). In Arabidopsis, ILITHYIA (ILA) is the only protein in the genome presenting high similarity to GCN1. Consistent with the model, ILA(GCN1) binds GCN2 in Arabidopsis, and *ila/gcn1* mutants are not able to phosphorylate eIF2 α (Wang et al. 2017; Faus et al. 2018). However, the behaviour in *gcn2* and *ila/gcn1* mutants differ under several abiotic stress situations. *gcn2* and wild-type seedlings are unaffected by mitochondrial dysfunction or boric acid, whereas both treatments lead to leaf yellowing and growth inhibition in *ila/gcn1*. Moreover, *ila/gcn1* mutant is more susceptible to *Pseudomonas* infection than wild-type and *gcn2* (Izquierdo et al. 2018). This indicates that ILA/GCN1 acts independent of GCN2 in stress situations and open a new pathway for translational regulation, governed by this protein, in plants.

Interestingly, the phenotypic differences between *gcn2* and *ila/gcn1* mutant extend to aspects of plant development. The weak alleles, like *ila3*, present leaf yellowness when grown in soil (Monaghan et al. 2010), have problems in chloroplast biogenesis and root development, and a dramatic alteration of its transcriptome at the seedling stage (Faus et al. 2018). The strong alleles, like *ila2*, are smaller than the wild-type, have yellow serrated leaves, and root growth and chloroplast deficiencies, and must be propagated as heterozygotes (Monaghan et al. 2010). This contrasts with the phenotype of *gcn2* mutants, which are indistinguishable of wild-type. All this indicates that ILA/GCN1 also acts independent of GCN2 in developmental processes. Transcriptomic analysis of the weak *ila3* allele showed a clear downregulation of genes related to photosynthesis, and a reduced expression of many genes involved in different aspects of defence responses, which would explain its susceptibility to biotic attacks. In opposite, antioxidant defences, protein folding, (marked by the increased expression of heat shock proteins (HSP), Lon proteases, responsible for the degradation of unstable proteins, and categories involving translation and RNA modification were enriched in *ila3* (Faus et al. 2018).

To gain a deeper understanding of the role of the ILA protein on plant development, a 2-D differential gel electrophoresis (2-DIGE) comparative proteomic assay to identify proteins differentially-expressed between wild-type and *ila2* seedlings was performed. Our results extend and complement the data obtained by transcriptomic for the weak *ila3* allele. The data confirm the link between *ila* developmental phenotypes and a correct ribosome assembly and protein cell homeostasis, as revealed by the differential expression of several heat-shock proteins and ribosomal proteins. Moreover, they confirm the chloroplasts defects of *ila* mutants, suggested by phenotypic and transcriptomic data.

Materials and methods

Sample material

Wild-type Col 0 and *ila2* (SALK_149084) lines were used in this study. *ila2* was obtained from NASC and genotyped using the insertion-flanking primers 5'-TGTTAGCCTCAGTCAAGTAC-3' and 5'-ATAGCCAGCTTCCCTTTCTC-3'. Seeds were sowed in 0.4% MS Salts agar plates, 1% sucrose, pH 5.7, and grown at 23 °C with a 16-h-light/8-h-dark cycle for up to three weeks for *ila2* (two weeks for wild-type) and equal-sized seedlings were collected for protein extraction.

Protein extraction and labelling

Seedlings were ground in liquid nitrogen and resuspended in 50 mM Tris pH 7.5, 1 mM PMSF, 0.2% β -mercaptoethanol and centrifuged for 20 min. 1% Trichloroacetic acid (TCA) was added to supernatant at 1:1 v/v. and incubated for 1 h. Samples were centrifuged for 20 min and washed three time with cold acetone.

Equal amounts (50 μ g) of protein samples were labelled using CyDyes DIGE fluors (Cy2, Cy3 and Cy5). Four replicates of wild-type and *ila2* were labelled in a dye-swap experiment using Cy3 and Cy5. A pooled sample containing all samples used in the experiment was labelled with Cy2 for internal control. Lysis buffer was added to make up the volume to 40 mL. Then, the pool was mixed with 40 μ L of isoelectrofocusing (IEF) rehydration buffer (8 M urea, 4% CHAPS, 0.005% bromophenol blue) containing 65 mM DTT and 1% IPG buffer pH 3–11, and it was loaded in the gel.

2D-electrophoresis

24-cm long strip with an immobilized pH gradient of 3–11 were hydrated overnight at room temperature with 450 μ L of IEF rehydration buffer, containing the reagents Destreak and Pharmalyte, pH 3–10. The CyDyes-labelled pool sample (150 μ g) was loaded on the hydrated strip. IEF was performed in an IPGphor unit at 20 °C and at a maximum current of 50 mA per strip at the following settings: 300 V for 1 h, an increasing voltage gradient to 1000 V for 6 h, an increasing voltage gradient to 8000 V for 3 h, before finally holding at 8000 V for a total of 32,000 Vh. After IEF, the strip was equilibrated separately for 15 min in 10 mL equilibration solution I (0.05 M Tris–HCl buffer, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, 2% DTT), followed by equilibration solution II (0.05 M Tris–HCl buffer pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide, 0.01% bromophenol blue) before being applied to the second dimension 12.5% SDS-PAGE gels. Gels were run at

20 °C by applying 2 W/gel for 30 min and 20 W/gel for the remaining 6 h in an Ettan DALT six unit. A running buffer of 25 mM Tris, pH 8.3, 192 mM glycine and 0.2% SDS was used.

Data analysis

Proteins were visualized using a Typhoon Trio scanner. The three images of each gel were processed with DIA (Differential in-gel analysis). DIA module was used for the spot detection, spot volume quantification and volume-ratio normalization of different samples in the same gel.

Protein spots that showed at least two-fold change in abundance between control and infected material and statistical significance using a Student's *t* test ($p < 0.05$) were considered differentially expressed.

Protein identification

Protein identification was performed by the Proteomic Service of the Universitat de Valencia. Spots of interest were excised from silver-stained gel, destained by 2–5 min washes with acetonitrile (ACN):water (1:1) and rehydrated with 50 mM ammonium bicarbonate for 5 min and 25 mM ammonium bicarbonate in 50% (v/v) ACN for 15 min. Samples were digested with 10 ng trypsin (50 mM NH_4HCO_3 , pH 7.8; 5 mM CaCl_2 ; 12.5 ng/ μL) (o/n at 37 °C).

The digestion was stopped with 7 mL of 0.1% Trifluoroacetic acid (TFA), and 1 μL was spotted onto the MALDI target plate. After air-drying the droplets at room temperature, 0.5 μL of matrix (5 mg/mL CHCA) (α -cyano-4-hydroxycinnamic acid, Sigma) in 0.1% TFA-ACN/ H_2O (1:1, v/v) was added and allowed to air-dry at room temperature. A 4700 Proteomics Analyzer was used for analyzing the resulting mixtures. Five of the most intense precursors (according to the threshold criteria: minimum signal-to-noise: 10, minimum cluster area: 500, maximum precursor gap: 200 ppm, maximum fraction gap: 4) were selected for every position for the MS/MS analysis. The MS/MS data were acquired using the default 1 kV MS/MS method and the MS and MS/MS information was sent to Protein Pilot v5.0 software. Protein assignment was achieved via BLASTtx, choosing those proteins with similarity over 90% based on sequence homology. The samples without a positive identification were analyzed by LC/MS/MS. Peptide separation was performed using an Ultimate nano-LC system and a QSTAR XL Q-TOF hybrid mass spectrometer. Samples (5 μL) were delivered to the system using a FAMOS autosampler at 30 $\mu\text{L}/\text{min}$, and peptides were trapped onto a PepMap C18 pre-column (5 mm $\times$ 300 mm i.d.). Peptides were then eluted onto the PepMap C18 analytical column (15 cm $\times$ 75 mm i.d.) at 300 nL/min and were separated using a 30 min gradient of 5–45% ACN. QSTAR

XL was operated in the information-dependent acquisition mode, in which a 1-s TOF MS scan from 400 to 2000 m/z was performed, followed by 3-s product ion scans from 65 to 2000 m/z on the three most intense doubly or triply charged ions. The MS/MS information was sent to ProteinPilot.

Results and discussion

2D-DIGE analysis reveals differentially-expressed proteins between wild-type and *ila2* seedlings

To investigate the impact that the loss-of-function of the ILA protein could provoke in Arabidopsis, the strong allele *ila2* was selected for comparison with wild-type. In this allele, a T-DNA is inserted in the 22nd exon of the gene, generating a non-functional protein without the C-terminal part (Monaghan et al. 2010). The strong phenotypes associated with this mutant (small size, yellow colour in emerging leaves, sterility in homozygous) confirm that the lack of this protein is affecting essential aspects of plant development. Given that the ILA protein has been involved in translational regulation, a comparative proteomic assay between wild-type and *ila2* seedlings will determine, first, to which extent the translational ability of the cells is affected in *ila2*, and second, will highlight the main effects of ILA loss-of-function. To avoid confusion due to the different developmental stages of the two genotypes, equal size, and not equal age, seedlings of wild-type and *ila2* were selected for protein extraction.

A side by side comparison of the 2D-DIGE images representing wild-type and *ila2* seedlings, as well as a composite image of both, is shown in Fig. 1a. A total of 1537 spots could be detected in this experiment, according to the DeCyder 2D software. The 2-DE profiles had a similar distribution pattern of spots in wild-type and *ila2*. In fact, only 125 spots (8.13%) were detected as differentially expressed between both genotypes, without an apparent bias towards any of them, with 81 more abundant in wild-type samples, and 44 more abundant in *ila2* samples. (Supplemental Table 1). Assuming that some of the spots will presumably resolve the same protein, reflecting different isoforms or posttranslational modifications (Carmona et al. 2019), the number of differentially expressed proteins between the two genotypes is expected to be even lower. These results indicate that the lack of ILA is not rendering a general arrest of protein translation in this mutant and that most cellular proteins needed for basic cellular functions are present in *ila2*. Besides, it indicates that protein accumulation in *ila2* is adjusted to the particular needs of this genotype, discarding an essential role of ILA in general aspects of the protein translation machinery.

As an initial approach to the nature of the differentially-expressed spots, eighteen of them were excised from

Fig. 1 2D-DIGE fluorescently labeled proteins. **a** Representative side-by-side comparison of gel images showing the differentially-labelled proteins from wild-type (Cy5) and *ila2* samples (Cy3), as well as pooled sample used for comparison (Cy2). **b** Differentially-expressed spots between wild-type and *ila2* seedlings are shown. Those that

were selected for extraction and MS analysis are marked with white circles. Volumetric quantification of representative spots of interest identified. Abundance values are expressed as the ratio obtained from volumetric image comparisons (*ila2*/wild-type)

2D-PAGE gels and subjected to identification via peptide-mass fingerprinting with MALDI-TOF-MS after homology search using ProteinPilot software (Table 1). For the selection, we mainly focused on the most differentially-expressed spots according to Supplemental Table 1, avoiding the very proximal or very faint ones. Six representative *ila2* overexpressed spots are displayed in Fig. 1b.

Down-regulated proteins in *ila2* confirm chloroplast and photosynthesis defects

ila mutants are defective in chlorophyll content and photosynthesis capacity (Monaghan et al. 2010; Faus et al. 2018). Moreover, chloroplasts of both the weak *ila3* and the strong *ila2* alleles present a poorly developed thylakoid membrane network, indicating a general defect in chloroplast biogenesis and function. This idea is reinforced by some of the proteins found differentially-expressed in *ila2*. Among the proteins with decreased protein expression in the mutant plants are carbonic anhydrase (CA1; At3g01500), and Rubisco activase (RCA; At2g39730). CA1 catalyzes the reversible hydration of CO₂ in higher plant tissues (DiMario et al. 2017). RCA regulates Rubisco function by facilitating the dissociation of

inhibitory sugar phosphates from the active site of Rubisco in an ATP-dependent manner (Portis et al. 2008). Additionally, other chloroplast proteins were found less expressed in *ila2* tissues, such as the PSII subunit PSBQ-2 (At4g05180), the small Rubisco subunit RBCS1A (At1g67090), the Calvin cycle intermediate phosphoribulokinase (At1g32060) and the Ferredoxin-NADP(+)-oxidoreductase 2 FNR2. Altogether, these results reinforce the observed chloroplastic defects of *ila* mutants and anticipate a putative cause of their defective plant development. The effects of a reduced level of FNR on plant development and on the structure and function of the photosynthetic machinery has been reported, *fnr* mutants presenting stunted growth and pale green leaves (Lintala et al. 2012). Similarly, mutants plants in the CA1 gene are smaller than the wild-type, and their development is strongly retarded (Fromm et al. 2016). Interestingly, and similar to *ila2*, plants lacking the two isoforms of the translation initiation eIFiso4G (*i4g1* and *i4g2*) display a defective phenotype that includes poor growth, lower chlorophyll levels, chloroplasts with lower grana stacking and lower fertility (Lellis et al. 2010; Chen et al. 2014). eIF4 factors interact with the mRNA and the 40S ribosome to assemble the pre-initiation complex. Similar to *ila2*, those plants present a

Table 1 List of differentially accumulated proteins identified between wild-type and *ila2* seedlings

Spot no.	Ratio <i>ila2</i> /wt	Acc. num	Protein pilot score	AGI name	Gene name	Prot. annotation	Functional classification
175	6.98	splQ8GUM2IHSP71_ ARATH	119.49	At4g37910	HSP70-9	Heat shock protein	Molecular chaperone
1097	6.36	splQ9FDZ9IRL212_ ARATH	4.01	At1g57660	RPL21E	60S ribosomal protein	Ribosomal protein
91	5.33	splP51818IHS903_ ARATH	39.68	At5g56010	HSP90-3	Heat shock protein	Molecular chaperone
1228	4.03	splQ03250IRBG7_ ARATH	26.22	At2g21660	GRP7	Glycine-rich RNA-binding protein	RNA transcription or processing during stress
661	3.99	splP49227IRL52_ ARATH	53.33	At5g39740	RPL5B	60S ribosomal protein	Ribosomal protein
1471	3.56	splQ8RXU5IR37A2_ ARATH	7.83	At3g60245	RPL37AC	60S ribosomal protein	Ribosomal protein
873	3.54	splQ8H156IRAN3_ ARATH	31.12	At5g55190	RAN3	GTP-binding nuclear protein	Nucleocytoplasmic transport
1308	3.08	splQ93VR4IML423_ ARATH	27.13	At1g24020	MLP423	MLP-like protein	Abscisic acid signalling pathway
1174	2.67	splQ93VP3IIF5A2_ ARATH	15.96	At1g26630	ELF5A-2	Eukaryotic translation initiation factor 5A-2	Protein biosynthesis
243	2.21	splQ8L7B5ICH60B_ ARATH	131.94	At2g33210	HSP60-2	Heat shock protein	Molecular chaperone
435	2.13	splQ9LUT2IMETK4_ ARATH	59.85	At3g17390	METK4	S-Adenosylmethionine synthase 4	Lignin biosynthetic process
664	– 5.12	splQ8W493IFNRL2_ ARATH	37.48	At1g20020	FNR2	Ferredoxin–NADP reductase	Photosynthesis
927	– 3.43	splP27140IBCA1_ ARATH	33.18	At3g01500	BCA1	Beta carbonic anhydrase	Photosynthesis
1289	– 3.24	splQ41932IPSBQ2_ ARATH	52.82	At4g05180	PBSQ2	Photosystem II subunit	Photosystem II assembly
702	– 3.16	splQ96262PCAP1_ ARATH	12.92	At4g20260	PCAP1	Plasma membrane-associated cation-binding protein	Response to abiotic stress
498	– 3.15	splP25697IKPPR_ ARATH	60.83	At1g32060	PRK	Phosphoribulokinase	Calvin cycle, photosynthesis
882	– 2.24	splP10795IRBS1A_ ARATH	26.01	At1g67090	RBCS-1A	Ribulose biphosphate carboxylase small chain	Calvin cycle, photosynthesis
427	– 2.22	splP10896IRCA_ ARATH	55.12	At2g39730	RCA	RubisCO activase	Activation of RuBisCO

reduced expression of CA1, RCA and PSBQ. In summary, the link between chloroplast missfunction and defective plant development is strengthened by the low expression of chloroplastic proteins in *ila2*. Moreover, the results obtained for *i4g* and also for *ila2* open a connexion between protein translation and chloroplast development.

Ribosomal proteins and heat-shock proteins are more expressed in *ila2* mutants

Remarkable, most of the identified proteins with increased protein expression in the *ila2* mutant plants belong to

two clear functional categories. Three heat-shock proteins, HSP90-3 (At5g56010), HSP70-9 (At4g37910) and HSP60-2 (At2g33210) were more increased in *ila2*. The spot identified as HSP70-9 (spot 1751) was the most differentially-expressed (6.98 fold), and the one identified as HSP90-3 (spot 91) is very proximal to five more spots (85, 90, 92, 93 and 99) which follow the same trend of expression in *ila2*, suggesting that at least some of them could be the isoforms HSP90-2 and HSP90-4 proteins (Krishna et al. 2001) or post-translational modifications of HSP90s (Mollapour et al. 2012) (supplemental Fig. 1). This indicates that *ila2* plants are constitutive subjected to

an internal stress condition where protein folding is disturbed. Interestingly, Faus et al. (2018) already reported an increase of HSP mRNAs in the weak allele *ila3* (Faus et al. 2018), reinforcing the idea of folding defects in *ila* plants. Curiously, among the proteins identified as more expressed in an *ila2* background, a 36% (4 out of 11) belong to the category of protein translation. One of them is the eIF5A-2 translational factor. eIF5 interacts with the 40S initiation complex to mediate hydrolysis of eIF2-GTP, a critical step in initiation complex formation, as the release of multiple eIFs, including eIF2-GDP, is necessary for the recruitment of 60S ribosomal subunit (Sharma et al. 2016). GCN1 facilitates the activation of eIF2 α kinase GCN2, which upon phosphorylation of eIF2 α , blocks eIF2-GTP hydrolysis (García-Barrio et al. 2000). Therefore, a non-functional GCN1 protein in *ila2* will not allow GCN2 to inhibit translation via eIF2 α phosphorylation. The overexpression of the eIF5 factor in *ila2* plants suggest that this initiation factor could compensate the defect in the GCN2-regulated inhibition of protein translation. In this regard, it has been reported that the effect of eIF2 phosphorylation can be mimicked by eIF5 overexpression, which turns eIF5 into translational inhibitor, thereby promoting the translation of GCN4 in yeast (Singh et al. 2006) and ATF4 in *Drosophila* (Kozel et al. 2016). For the first time, this evidences a similar mechanism orchestrating in plants, although the existence of GCN4/ATF4-like genes in Arabidopsis is unknown.

In addition, three ribosomal proteins, RPL21E (At1g57860), RPL5B (At5g39740) and RPL37A2 (At3g60245) were also overexpressed in *ila2*. Disturbance of ribosome biogenesis by distinct external and/or internal stimulations, (like malfunction of genes required for this process), results in ribosomal stress, leading to accumulation of ribosome-free form of RPs (Zhou et al. 2015). These results suggest that, under certain conditions or developmental stages, ILA1 could play a role in ribosome assembly, and its loss-of-function causes protein folding stress and free ribosomal protein accumulation, that will eventually perform ribosome independent functions (Zhou et al. 2015). Again, this is the first evidence of ILA affecting ribosomal stress and accumulation of RPs.

In conclusion, these results suggest that ILA is a central player in GCN2-mediated and GCN2-independent protein translation and ribosome assembly, and its loss-of-function causes a decrease in photosynthesis proteins, protein folding stress and free ribosomal protein accumulation.

Author contribution statement IF and RN performed the experiments. ST performed protein analysis. Jose Gadea and Regina Niñoles design the experiment. José Gadea wrote the manuscript.

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