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

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Abbreviations: GCN (General Control Non-derepresible)

Summary

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.

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 (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 & 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 & 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 (Johnson *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).

In this work, we show that ILA is able to bind AtGCN2 through its conserved C-terminal interaction domain, and it is required for phosphorylation of eIF2α, confirming that the formation of a ILA-GCN2 complex needed for GCN2 activation is also functioning in plants. However, we present evidence suggesting that the inability to phosphorylate eIF2α is not the cause of the phenotypes observed in the *ila* mutants, probably indicating for the first time GCN2-independent roles of GCN1 homologs. In this study, the first steps towards the understanding of this GCN2-independent role of ILA in plant development are taken.

Results.

ILITHYIA interacts with AtGCN2 through the C-terminal domain and is needed for eIF2α phosphorylation.

The Arabidopsis ILA protein contains the domains known to be relevant for GCN1 function, and also for interaction with GCN2. A BLAST search indicates that the ILA gene presents a 43% identity and 69% similarity in amino acids 1417 to 1720 with the EF3-like domain (similar to the translation elongation factor 3) found in the GCN1 yeast gene, including the GCN20-interacting region (Marton *et al*. 1993, Figure 1a). A BLAST search using the GCN2-binding region of the yeast GCN1 protein identified by Sattlegger and Hinnebusch (amino acids 2052-2428) highlighted a 36% identity and 54% similarity with the C-terminal region of ILA, comprising the amino acids 2139-2501 and including the conserved arginine residue (R2347 in ILA) within the consensus sequence needed for interaction with GCN2 (Pereira *et al.* 2005). The AtGCN2 protein has all the functionally distinct domains conserved in other GCN2 proteins including the minimal essential region in the N-terminal region needed for interaction with GCN1 (Zhang *et al.* 2003, Figure 1a). This N-terminal domain contains the topology characteristic of the GCN1-interacting domain of the GCN2 genes (Nameki *et al*. 2004).

To experimentally confirm association of ILA with GCN2 in Arabidopsis, the C-terminal part of the ILA protein (ILA-C-term), comprising amino acids 2098-2696, and including the eventual GCN2-interacting region, was translationally fused to the YFP^c protein in the pYFC43 vector (Belda-Palazon *et al*. 2012). The complete AtGCN2 protein was fused to the YFP^N protein in the pYFN43 vector. We then assayed the eventual association by bimolecular fluorescent complementation (BiFC). Additionally, we also fused AtGCN2 to YFP^C to assay GCN2 dimerization. *N. benthamiana* leaves were also transiently transformed with different combination of control constructs as shown in Supplemental Figure 1. Coexpression of YFP^N-GCN2 and YFP^C-GCN2 reconstituted YFP along the cell perimeters, suggesting that GCN2 also dimerizes *in planta* in the cytoplasm (Supplemental Figure 1). Confirming the recent results of Wang *et al*, 2016, co-expression of YFP^N-GCN2 and YFP^C-ILA(C-term) yields a detectable signal along the edges of the cells, confirming that ILA is at least close associated *in vivo* with AtGCN2, presumably in the cytoplasm.

It has been reported that the GCN1-interacting domain of GCN2 is well conserved among the GCN2 proteins. The *Drosophila* GCN2, for instance, can interact with the yeast GCN1/GCN20 complex (Garcia-Barrio *et al*. 2000), suggesting an evolutionary conservation mode of GCN1/GCN2 interaction. In yeast, the transcription factor GCN4 is under translational regulation and is synthesized under conditions that lower the amounts of active ternary complex (eIF2-GTP-$tRNA^{Met}$), such as when eIF2α is phosphorylated. Thus, yeast cells deficient in eIF2α phosphorylation should not translate GCN4 efficiently. If ILA is the GCN1 ortholog of Arabidopsis, a truncated ILA protein containing the GCN2-interating domain could compete with the *Saccharomyces* GCN1 for GCN2 binding and could prevent activation of GCN2 in a yeast assay. To test this hypothesis, we generated an hemagglutinin (HA)-tagged truncated ILA protein under the control of the GAL promoter, and expressed it in a yeast strain containing the entire GCN4 5′-untranslated region driving expression of the LacZ gene. After induction, the transformed strain was able to overexpress the truncated ILA protein, as shown by Western blot (Supplemental Figure 2). As expected, under amino acid starvation (-aas), GCN4 expression increased in the yeast strain transformed with the empty vector (Figure 1b); however, this increase was abolished in the yeast strain harboring the truncated ILA protein, suggesting a dominant negative effect in an endogenous pathway requiring functional GCN1 (likely the GCN1-GCN2 interaction) and disrupting the regulatory control exerted over GCN4.

Finally, we approached the involvement of ILA in the activation of GCN2 by assaying the phosphorylation of the GCN2 substrate eIF2α in *ila* and *gcn2* mutants. The *ila3* allele harbors a T-DNA insertion in the very C-terminal part of the gene (54th exon), and disrupts presumably the region for GCN2-interaction. *Ila3* was treated with 1mM glyphosate, known to phosphorylate eIF2α in Arabidopsis in a GCN2-dependent manner (Faus *et al*. 2015). As shown in Figure 1c, exposing Arabidopsis seedlings to glyphosate stress results in eIF2α phosphorylation as detected by on Western blots performed using phosphospecific antibodies for eIF2α (P-eIF2α), possibly indicating activation by GCN2. As expected, no phosphorylation was observed in the *gcn2* mutant. Supporting the recent data of Wang *et al*. 2016 with chlorsulfuron on other *ila* alleles, no P-eIF2α was detected in *ila3* seedlings exposed to glyphosate.

Photosynthesis and root growth are affected in *ila* mutants but not in gcn2

ila3 homozygous lines develop a chlorotic phenotype in emerging leaves. The phenotype is more dramatic in the *ila1* and *ila2* alleles, which present T-DNA insertions in the central region of the gene, disrupting the 22nd and 24th exons, respectively (Monaghan and Li, 2010). Curiously, no eIF2α phosphorylation was observed in *ila3* (Figure 1c) or *ila2* (Supplemental Figure 2) alleles, indicating that the inability to perform this posttranslational modification is not the only cause for the developmental defects of the strong alleles.

To confirm this observation, a comparison of the phenotypes observed in both *ila3* and *gcn2* mutant lines is presented here. To our knowledge, the only available homozygous Arabidopsis mutant line in GCN2 (GT8359) is in the Landsberg *erecta* (Ler) accession (Zhang *et al*. 2008). In this mutant, a Ds transposon is inserted in the first intron of GCN2, and it has been suggested to lead to a weak mutation (Wang *et al*. 2016). To compare the phenotype of both mutants in the same genetic background, a new *gcn2* insertion mutant line in the Columbia accession (Col-0) was isolated. The sequenced SALKseq_032196 line contains two T-DNA insertions in gene regions: one in the 15th exon of AT3G59410 (GCN2), disrupting the kinase domain, and the other one in the third intron of AT5G18610. Starting from a segregating population of the mutant line, the progeny was screened for wild-type genotypes in AT5G18610 and homozygosity for the insertion in GCN2. The new allele was named *gcn2-2,* and the original Landsberg allele described by Zhang, 2008 (GeneTrap line GT8359) was renamed *gcn2-1*. Western blot analysis of *gcn2-2* seedlings treated with UV-C demonstrates that this mutant line is unable to phosphorylate eIF2α.

Complemented *gcn2-2* lines expressing GCN2 under the constitutive 35S promoter could phosphorylate eIF2α after UV-C treatment, indicating that the inability of *gcn2-2* to phosphorylate eIF2α is GCN2-dependent (Supplemental Figure 4c). A chimeric mRNA containing the T-DNA is transcribed in the *gcn2-2* allele, as observed in RT-PCR reactions, likely yielding a non-functional protein unable to phosphorylate eIF2α. (Supplemental Figure 5). As the T-DNA is disrupting the kinase domain, we consider *gcn2-2* a knock-out allele.

In contrast to *ila* mutants, *gcn2-2* grow normally and does not develop a chlorotic leaf phenotype, nor any of the other developmental and fertility defects found in the strong *ila* alleles (Supplemental Figure 4a, b).This result suggests that the phenotype observed in *ila* alleles is not due to the inability to phosphorylate eIF2α via GCN2. The root phenotype of *ila* and *gcn2* mutants also indicates that both genes could also be performing different functions. As shown in Figure 2a, root elongation is affected in *ila* mutants, a phenotype that is not observed in *gcn2-2*. This effect is more dramatic in the strong *ila2* allele, where the primary root length presents a strong reduction after 20 days growing vertically on MS plates (Figure 2b). Again, these results indicate that the cause of the root phenotype is not due to the inability to phosphorylate eIF2α, and further suggest a novel eIF2α/GCN2-independent role for ILA.

The chlorotic phenotype of the young *ila* leaves prompted us to analyze the effects of these mutations on various parameters associated with photosynthesis on mature and young leaves of *ila3* and *gcn2-2* plants as compared with wild-type (Col-0) of the same developmental age. As expected by the previous data from Monaghan and Li 2010, the young leaves of *ila3* have significantly lower chlorophyll content (SPAD) than the equivalent Col-0 or *gcn2-2* leaves (Table 1) and mature *ila3* leaves have recovered normal levels of chlorophyll, reaching that of the adult Col-0 and *gcn2-2* leaves. The photosynthetic capacity of the three genotypes was similar in mature leaves, since no differences were found for photosynthetic rate, quantum efficiency of PSII, and maximum photochemical efficiency (Table 1). It is noteworthy that *ila3* plants show higher transpiration rates probably derived from a higher stomatal conductance. In young leaves, *gcn2-2* displays similar photosynthetic parameters than Col-0; *ila3*, by contrast, showed a significant decrease in photosynthetic rate and actual quantum efficiency of PSII. An increase in substomatal CO_2 concentration of *ila3* suggests biochemical limitations to photosynthesis in this genotype. In addition, a slight decrease in the maximum photochemical efficiency (F_v/F_m) was observed. This decrease was related to a decrease in the minimal Chl a fluorescence in the dark adapted state, Fo (Table 1), which points to damage in the antenna pigments. In addition, the reduction in the

maximal Chl a in the dark state Fm (Table 1) indicates that the photochemistry of PSII and its ability to reduce the primary acceptor Q_A was affected in the *ila3* plants. All these data suggest chloroplast defects in the *ila3* mutant, unexpectedly not shared by *gcn2-2*.

Chloroplast development is affected in young leaves of *ila* mutants.

Analysis of chloroplasts by electron microscopy indicates that thylakoid organization is affected in young *ila2* and *ila3* leaves. Col-0 young leaves contained fully developed ovoid chloroplasts with internal thylakoid membranes densely stacked into grana layers (Figure 3a). In contrast, *ila* chloroplasts contained a poorly developed thylakoid membrane network with more luminal area between the thylakoid membranes as compared to wild-type ones of the same age (Figure 3b,d). The number of chloroplasts, size and envelope membrane structure, however, appears normal. In more developed leaves, these differences between wild-type and *ila* chloroplast are attenuated (data not shown). In general, *gcn2-2* chloroplasts presented an appearance more similar to wild-type, with a dense organized thylakoid structure and well stacked grana system (Figure 3c). These results suggest that the ILA protein is necessary for the correct development of the thylakoid network in the chloroplasts, a role that seems independent of GCN2.

The *gcn2 ila3* double mutant maintains the developmental phenotypes characteristic of *ila3* single mutant.

The proposed role for GCN1 is to position GCN2 on the ribosome in such a way, that uncharged tRNAs can be transferred from the ribosome to GCN2 under stress conditions (Sattleger and Hinnebusch, 2000). A possible explanation for the *ila* phenotype would be that GCN2 would not be recruited anymore to the ribosome, and potencially free kinase would then be prone to phosphorylate other substrates, resulting in unexpected phenotypes as the observed defect in chloroplast development and root growth. We crossed *ila3* with the *gcn2-2* line, in the Columbia accession, and genotyped an F2 population for double mutants *ila3*/*gcn2-2*. The phenotype of the roots and emerging leaves in two- to four-weeks old seedlings was characterized. As shown, *ila3/gcn2-2* mutant lines retain the chlorotic phenotype (Figure 4a) and root defects (Figure 2a) characteristic of the *ila3* single mutant. Photosynthetic measurements show that the double mutant retains the lower chlorophyll content and photosynthetic defects already presented for *ila3* (Figure 4b). This result suggests that these *ila* phenotypes are specific to the lack of the ILA protein in the

cell, performing a GCN2-independent function, and not to an indirect effect on a eventually free GCN2 protein in an *ila* background.

Transcriptome analysis of the *ila3* mutants.

To gain insight into the molecular mechanisms affected by the lack of the ILA protein, the transcriptome was compared of two-week old wild-type and *ila3* seedlings. 113 genes were considered up-regulated and 324 down-regulated in the *ila3* mutant (Supplemental Table 1). Gene set enrichment analysis indicates that many categories involved in defense response were enriched among the genes less expressed in *ila3* (Figure 5, Supplemental Table 2). Interestingly, we found that *ila3* mutation is affecting the basal expression of genes known to participate in different aspects of defense responses. Among others, a cluster of cysteine-rich receptor-like protein kinases (CRKs), which play important roles in the regulation of pathogen defense, are low expressed in *ila3;* the same behavior is observed for the EDS1b gene, 82% to the canonical EDS1a, a key gene in the defense response, directing both the salicylic acid (SA)-dependent and SA-independent branches of basal resistance and systemic acquired resistance (SAR) (Feys *et al*. 2005). Moreover, the recently identified EDS1-dependent SAR-regulators AED1, LLP1, PNP-A, PR2, and AED15 (Breitenbach *et al*.2014) were also less expressed in *ila3* mutants. The same trend was found, among others, for the well-studied defense-related BDA1, WAK1, MPK11 or PCC1 genes, implicated in different aspects of plant defense (Supplemental Table 1).

The GO category covering the light harvesting components of the photosynthesis was also found enriched among the genes less expressed in the *ila3* mutant (Figure 5, Supplemental Table 2). This category includes components of the antenna system, such as the light-harvesting proteins (Lhcb1.1, Lhcb2.2, 2.3 and 2.4), and chlorophyll binding-protein CP22, belonging to the photosystem I (PSII), but also the antenna protein Lhca2 and PsA, the reaction center of the photosystem I, all of them having the expression affected in the *ila3* mutants. Other photosynthetic-related genes less expressed in *ila3* include the *POR A* gene, encoding a protochlorophyllide oxidoreductase A, a key enzyme in chlorophyll biosynthesis, the FED2 gene, encoding the major leaf ferredoxin, or the AtpOMT1 gene, encoding the oxalacetate/malate transporter involved in the dissipation of excess electrons to protect the photosynthetic apparatus. Finally, root morphogenesis category was also enriched upon the genes less expressed in *ila3* (Supplemental Table 2), suggesting why this cellular process is affected in this mutant.

Among the biological processes that were enriched upon the genes more expressed in the mutant *ila3* plants, we found processes involving the superoxide ion, including the expression of the chloroplastic (CSD2) copper/zinc and its chaperone ATCCS, as well as the cytosolic superoxide dismutase (CSD1). Another noteworthy category enriched among the genes more expressed in *ila3* is protein folding, marked by the increased expression of heat shock proteins (HSP) such as two HSP20-like proteins, the chloroplastic DJA4/HSP40 protein, or the HSP70. The expression of the proteases Lon3 and Lon4, responsible for the degradation of damaged and unstable proteins, was also increased in *ila3*. Categories involving translation, RNA modification or protein import were also enriched upon the genes less expressed in *ila3* (Supplemental Table 2), indicating that these molecular and cellular processes could also be affected in the mutant.

DISCUSSION

GCN2 needs ILA for eIF2α-phosphorylation

There is a strong evidence for the universality of the basic biology of all eukaryotes. Proteins essential for viability and/or that interact with other proteins are more likely conserved, and this seems to be the case for the proteins involved in the phosphorylation of the translational initiation factor eIF2α. Conserved from yeast to human, the kinase GCN2 has also been identified in Arabidopsis, and some of the mechanisms concerning its molecular function have been demonstrated in plants (Li *et al*, 2013; Zhang *et al*, 2008). The other two main components of the GCN system (GCN1 and GCN20) have been proposed to exist in Arabidopsis. However, links between these three genes are dispersed and not conclusive regarding the relationship with eIF2α-phosphorylation. ILITHYA (ILA) is the only protein in Arabidopsis sharing homology with the ScGCN1 gene, and was involved in plant immunity (Monaghan and Li, 2010). The gene SCORD5 (AtABCF3) was isolated in a genetic screen to rescue the virulence of COR-deficient mutant bacteria and it was shown to have homology to ScGCN20, and proposed to be functionally linked to ILITHYA (Zeng *et al*, 2011). GCN20 is a positive regulator of GCN2 in yeast, and it has been proposed to stimulate GCN2 activation by uncharged tRNAs (García-Barrio *et al*, 2000). SCORD5, however, belongs to a family of ATP-binding cassettes (ABC)-transporters and at least five genes share more than 90% sequence similarity with ScGCN20 (Sanchez-Fernandez *et al*.2001); neither ILA nor SCORD5 were tested for functional association with the GCN2 kinase in these studies. The recent results from Wang *et al* and ours showing that AtGCN2 interacts with the putative GCN2-interacting domain of

ILA and that the *ila* mutants are not able to phosphorylate eIF2α (Figure 1) contributes to reinforce the idea of a functional GCN pathway in Arabidopsis. The presence of a eFE3-like domain in the middle portion of the ILA protein also suggest its binding to the ribosome, where GCN1 is known to promote GCN2 function (Marton *et al*, 1997). The similarities with the yeast system are not complete yet, however, as any homolog of the transcription factor GCN4 has been found in Arabidopsis so far. The relevance of eIF2α-phosphorylation-mediated translational arrest in plants is still under discussion (Immanuel *et al*. 2012).

A new eIF2α-independent function for ILA/GCN1.

The difference in phenotype between *gcn2-2* and *ila3* mutant plants suggests that promoting phosphorylation of eIF2α by GCN2 is not the only function of the ILA protein. The existence of a stronger allele (*ila2*), with a central T-DNA insertion, showing a more severe phenotype than *ila3*, suggests that the eIF2α-independent function of ILA could be mediated by this central region. The disruption of this putative ribosome-binding domain could be affecting the ribosome-binding properties of the protein or the binding to other effectors involved in translation-related or unrelated functions, independent of eIF2α phosphorylation. Indeed, this EF3-like domain in GCN1 also constitutes the binding domain for the N-terminal part of GCN20 in yeast (Marton *et al*, 1997). The remainder of GCN20 shows strong similarity to the C-terminal part of eEF3, encompassing two ATP-binding cassettes (ABC), so the GCN1/GCN20 complex would retain the ribosome-binding properties characteristic of eEF3 in yeast. Interestingly, one of the homolog proteins of GCN20 in Arabidopsis (SCORD5) also presents a chlorotic phenotype in the emerging leaves, and both the ILA and SCORD5 proteins are required for bacterium-triggered stomatal closure response (Zeng *et al*, 2011), suggesting a functional link between the two proteins. It would be interesting to determine whether these correlations in phenotype obey to the same cellular defect and whether the *scord5* mutant is able to phosphorylate eIF2α.

GCN1 has been defined as a scaffold protein. It could then be hypothesized that ILA contributes to modulate kinase specificity by recruiting GCN2 to the ribosomes. In an *ila* background, GCN2 would be free to phosphorylate other proteins, causing off-target effects, evidenced in the observed *ila* phenotypes. This hypothesis was discarded, as double mutant *ila3 x gcn2* still presented the characteristic *ila3* phenotypes (Figures 2 and 4). Supporting these results, no *ila3*-like phenotypes were observed in GCN2

overexpressing transgenic wheat (Byrne *et al*, 2012), clearly indicating that the *ila* phenotypes are not due to an artefactual action of GCN2.

HEAT-repeat proteins are involved in a great diversity of processes mediating protein-protein interactions. GCN1-like proteins could then bind more proteins than those known so far. In one study in humans, the GCN1 homolog was found in the core of the spliceosome CDC5L complex (Ajuh *et al*, 2000). In plants, this complex shares some proteins with the MOS4-associated complex, involved in the regulation of plant immune responses. However, no evidence was found of ILA being a component of this complex in Arabidopsis (Monaghan and Li, 2010). A GCN1 homolog is also involved in *C. elegans* morphogenesis, where loss-of-functions suppress the defect in semaphorin mutants. Semaphorin-mediating signaling determines tail morphogenesis by decreasing eIF2α phosphorylation. Interestingly, knock-down of GCN2 do not affect tail morphogenesis, suggesting that this GCN1 homolog could have a GCN2-independent role in this process as well (Nukazuka *et al*, 2008). This putative GCN2-independent function of GCN1 would be still linked to eIF2α phosphorylation, as this posttranslational modification is determinant for semaphoring signaling. As state above, eIF2α do not seem to be mediating the *ila* phenotypes in Arabidopsis, suggesting a new eIF2α independent function for the ILA protein.

The consequences of ILITHYA mutation in chloroplast biogenesis

The chlorotic phenotype of *ila* mutants suggested that ILA is another protein required for proper chloroplast function. Photosynthetic measurements and analysis of chloroplasts suggest that thylakoid organization is affected and photosynthesis is impaired in young leaves of *ila* (Figure 3 and Table 1). The chloroplast structure, however, is maintained, and chloroplasts are not as severely damaged as they are in other mutants deficient in thylakoid formation, as *AtTerC* or *Thf1,* where the thylakoid matrix is completely disrupted (Kwon and Cho, 2008, Wang *et al*, 2004).

In *ila* mutants, chloroplast function recovers as the plants age. Many reports of chloroplast-linked mutations are describing a dramatic effect in the early stages of leaf development that is overcome in later stages (Jarvis *et al*. 1998; Wang *et al*. 2004). The suggested explanation has been a mechanism in the plant that compensates the reduction of components important for chloroplast development. In the case of ILA, it is difficult at this point to predict at which level the defect will be counterbalanced, but, given that ILA do not present homology to any other protein in the Arabidopsis genome, it seems plausible that the

targets for compensation will be the effects of ILA disruption, and not a substitution of ILA molecular function.

The increased expression in *ila3* tissues of genes involved in removal of superoxide could indicate a context of oxidative stress in *ila* mutants (Alscher *et al*. 2002). A possible cause could be the decreased expression of the POR A gene, involved in the light-dependent reduction of the protochlorophyllide (Pchlide) to chlorophyllide, that could indicate a defect in the ability to prevent photooxidative stress, as the maintenance of an optimal Pchlide:POR ratio is essential because free Pchlide (not bound to POR) operates as a photosensitizer upon light exposure causing oxidative stress (op den Camp *et al*. 2003). Curiously, both the Arabidopsis *porB/porC* mutants display highly chlorophyll-deficient phenotypes (Paddock *et al*. 2010). In the variegated *var2* mutant, involved in the degradation of thylakoid membrane proteins, the green sectors mainly accumulates ROS, while the white ones are ROS-free but are the ones that express antioxidant enzymes. These results imply that variegated sectors might be maintained positively through the expression of genes related to oxidative stress detoxification (Miura *et al*. 2010). The induction of other ROS-scavenging systems to protect damaged chloroplasts has also been described in Arabidopsis *fnr* mutants, that also display a highly chloroplast-deficient phenotype (Lintala *et al*. 2007, 2012). A similar mechanism could also be occurring in young *ila* leaves as a common response to protect defective tissues that result from the mutation.

A number of heat shock proteins were up-regulated in *ila3*. This could also be understood as another response to safeguard damaged tissues consequence of mutations (Rajan and D'silva. 2009). HSPs have been found to mediate chloroplast and mitochondria import, including cytosolic HSP70s as the one found induced in *ila3* mutants (Flores-Perez and Jarvis, 2013). Interestingly, the phenotype of plants deficient in the Toc 33 gene, involved in protein import, resembles that of the *ila* mutants. It would be interesting to assay whether protein import to organelles or other processes involving HSPs are working properly in *ila* mutants. The induction of HSP proteins was already reported in some mutants defective in chloroplast development. The white sectors of the variegated mutant *var2* of Arabidopsis reveals induction of numerous heat-shock proteins (Miura *et al*. 2010). Similarly, mutants in the *clpr2* subunit of the Clp protease complex also present delayed chloroplast and plant development with a pale green phenotype. This protease complex is part of the protein homeostasis network in the chloroplast, and *clpr2* mutants also overexpress a battery of proteins involved in folding and import, including several chloroplastic HSPs (Lintala *et al*. 2007). It is important to remark that the reduced expression of these genes is not

happening in *gcn2* mutants. Transcriptomes of wild-type and *gcn2-1* seedling did not reveal great differences in gene expression between both genotypes (Faus *et al*. 2015). Therefore, these altered gene expression patterns are presumably independent of eIF2α phosphorylation.

ILA contribution to plant immunity.

Non-host, basal defense and systemic acquired resistance (SAR) are affected in *ila* mutants (Monaghan and Li, 2010). Numerous genes related to these defense responses are less expressed in *ila3* (Supplemental Table 1). Thus gene activation under an eventual pathogen attack could be also compromised in these mutants. The chloroplast is the site of biosynthesis of Salycilic acid (SA) (Wildermuth *et al*. 2001), However, *ila3* mutants were able to accumulate SA upon *P. syringae* infection, and retain the ability to perceive SA signals, suggesting that the SAR defects in *ila* could be independent of SA (Monaghan and Li, 2010). This data will suggest that the defective chloroplasts of *ila3* still retain the ability to synthesize SA and trigger defense responses. The accumulation of SA was, however, slightly lower in *ila3* than in wild-type plants upon pathogen infection (Monaghan and Li, 2010). This could explain the constitutive lower expression of many SA-dependent genes identified in our transcriptomic experiment. Further experiments monitoring expression after pathogen infection will be necessary to confirm if the activation ability of SA-dependent genes in *ila* mutants is complete.

Functional chloroplasts are also necessary for the establishment of a full cell death response. Cell death responses following pathogen infection relay on the oxidative burst triggered by the infected cells after recognition of specific bacterial effectors. In recent years, chloroplast-derived ROS have been implicated in different aspects of this plant defense. Chloroplast ROS build-up and cell death were significantly reduced in *Xanthomonas campestris vesicatoria*-inoculated Arabidopsis plants expressing plastid-targeted flavodoxin, indicating a chloroplastic origin of the cell death events following non-host response (Zurbriggen *et al*. 2009). These results indicate that chloroplast-generated ROS play an important role in triggering and/or in the execution of cell death during this non-host interaction. Moreover, light is necessary for the complete deployment of a HR response (Mur *et al*. 2008), it is abolished or delayed in the dark (Liu *et al*. 2007) and chloroplastic ROS control the expression of nuclear-encoded genes for defense response (Fernandez and Strand 2008). The defective chloroplasts of *ila* mutants could be unable to set up a complete ROS-dependent response under pathogen infection that will affect the ability to orchestrate cell death responses. Moreover, the activation of antioxidant genes discussed above could also

mitigate the production of chloroplastic ROS. The basal expression of genes involved in cell death responses is already lower in *ila3*, indicating that the mechanisms of gene activation mediated by chloroplastic ROS could be impaired. For instance, it has been described that loss-of-function mutations in BDA1, nine times less expressed in *ila3*, suppress the constitutive defense responses in snc2-1D npr1-1 and result in enhanced susceptibility to bacterial pathogens. In contrast, a gain-of-function allele of *bda1* was found to constitutively activate cell death and defense responses (Yang *et al*. 2012). Similarly, overexpression of several CRKs, including CRK6, six time repressed in *ila3,* enhance a pattern-immunity-response (PTI) and resistance to virulent bacteria *Pseudomonas syringae* pv. tomato DC3000 (Yeh *et al*. 2015). The susceptibility of *ila* mutants to pathogen infection could again be a consequence of the defect in chloroplast biogenesis of these mutants.

Conclusions

Based in the results presented in this study, we confirm that ILA is the homolog gene of the yeast GCN1, and that its presence is essential for GCN2-dependent eIF2α phosphorylation. However, it becomes apparent that additional functions will have to be assigned to the ILA protein in the future. Given that this differences in phenotype have not been observed so far in the yeast GCN system, it seem plausible that this new function could have evolved in multicellular organisms, and could even be unique to plants. We argue that many of the apparently distinct phenotypes of the *ila* mutants could be consequences of a defect in chloroplast biogenesis. This would explain why this new function has remained undiscovered in the yeast system, and opens the door for the observation of *gcn1* mutants in other organisms. Understanding important biological processes such as translational regulation, organelle biogenesis or protein import is at stake.

Materials and Methods

Plant Material

Arabidopsis thaliana plants were grown on a soil mix of 25% perlite, 25% vermiculite and 50% peat moss, in environmental growth chambers under long-day (16 hours light at 21°C and 8 hours dark at 19°C) photoperiod cycle, with a light intensity of 150 µmol m^{-2} s^{-1}. The following genotypes were used in this study: wild-type Col-0, *ila3* (SALK_ 041123), *ila2* (SALK_ 149084), *gcn2-1* (Genetrap line

GT8359, Cold spring Harbor Laboratory, also see Figure 1), *gcn2-2* (SALK_032196), and *ila3* x *gcn2-2* double mutant. SALK lines were obtained from NASC and genotyped using the insertion-flanking primers 5′-TGTTAGCCTCAGTCAAGTAC-3′ and 5′-ATAGCCAGCTTCCCTTTCTC-3′ for *ila2* and 5′-CACAAGGACTAACCTTGTAG-3′ and 5′-GAAGTTACTAGCGAGCAAGC-3′ for *ila3*. *ila2* mutants are sterile and must be propagated as heterozygotes. For *gcn2-2* genotyping, see paragraph below. For complementation, GCN2 complete open reading frame was amplified from Col-0 cDNA using the following primers: 5'-ACCATGGGTCGCAGCAGTTC-3' and 5'-TTATTAGCTCCAAACAGAGGGGTTTCT-3' and cloned in pCR8/GW/TOPO vector, and later in pMDC32 as destination vector (Curtis and Grossniklaus, 2003). The final construct was introduced into *Agrobacterium* C85 and *Arabidopsis gcn2-2* plants were transformed by floral dipping. Transformants were checked by PCR using GCN2-specific primers.

Nicotiana benthamiana seeds were sown on a soil mix of 50% vermiculite and 50% peat moss and grown for four weeks in controlled greenhouse conditions under long-day photoperiod cycles (16 hours light/8 hours dark) at 22°C±1°C.

***gcn2-2* genotyping**

SALKseq_032196 was ordered from NASC (Nottingham Arabidopsis Stock Centre) and genotyped using primers flanking the T-DNA insertion (5'-GGACAATAATCTTGAGTCGAC-3' and 5"-CCCTTTCAGCTTAGCTTCGGAGAT-3") and the T-DNA specific primer Lbc1 5"-TGGACCGCTTGCTGCAACTCT-3". Since the original had an insertion also in the AT5G18610 gene we genotyped for the absence of this second insertion with primers 5'-AGGGACTTAGCTTCGGAGAT-3' and 5'-GGACCTCGTCGAGACTTTG-3') and the T-DNA specific primer Lba1 5'-TGGTTCACGTAGTGGGCCA-3'.

For RT-PCR reaction over *gcn2-2*, the following primers were used: FP1: 5'-GGACAATAATCTTGAGTCGAC-3'; RP1: 5'-CCCTTTCAGCTTCAGGTTAG-3'; FP2: 5'-GGTTTTGTGGAGATGCAGATC-3'; RP2: 5'-TTAGCTCCAAACAGAGGGGTTTCT-3' and FP3: (LBb1.3 recommended for Salk lines genotyping): 5'-ATTTTGCCGATTTCGGAAC-3'.

Photosynthetic measurements

Simultaneous gas exchange and chlorophyll fluorescence measurements were performed 2007 with a LI-6400 (LICOR, Nebraska, USA) as described in Flexas *et al*. 2007. Instantaneous determinations of net CO_2 assimilation rate (A_N), stomatal conductance (G_s), transpiration rate (E) and substomatal CO_2 concentration (C_i) were carried out at steady-state conditions under saturating light (1000 µmol m^{-2} s^{-1}), a vapour pressure difference (vpd) between 1 and 2 kPa and 400 ppm CO_2. The actual photochemical efficiency of photosystem II (PhiPS2) was determined by measuring steady-state fluorescence (F_s) and maximum fluorescence (F_m') during a light-saturating pulse (8000 µmol m^{-2} s^{-1}) (Genty *et al* 1989). Maximal photochemical efficiency (F_v/F_m) on dark adapted leaves was measured with a MINI PAM fluorometer (Walz, Effeltrich, Germany). SPAD values were measured with a chlorophyll meter SPAD-502 (Konica Minolta, Osaka, Japan). One measurement per plant was taken, and for each genotype, 8 to 10 different plants were measured.

Bimolecular fluorescence complementation (BiFC) assay

BiFC assays were performed transiently using *Agrobacterium*-mediated co-infiltration of 4-week-old *N. benthamiana* leaves and *Agrobacterium* strain C85 harboring the appropriate plasmids. To suppress gene silencing, *A. tumefaciens* cells expressing the p19 protein of the tomato bushy stunt virus were used in the co-infiltration procedure. Overnight grown cultures of *A. tumefaciens* of about 2.0 OD_{600} units were collected and resuspended in similar volume of infiltration buffer ($MgCl_2$ 10 mM, MES 10 mM pH 5.6, acetosyringone 200 mM) and incubated at 28ºC for 4 hours. A mixture of *Agrobacterium* strains containing the two constructs and the p19 plasmid at OD_{600} 1.0:1.0:1.0 was prepared for co-infiltration into the abaxial side of *N. benthamiana* leaves with a needleless syringe. Epidermal cell layers of at least two transformed leaves of 3–4 plants of similar age were assayed for fluorescence under confocal microscope 2 days after infiltration. Excitation/emission of 500-530 nm (gain 850, 2% transmission laser) (YFP fluorescence) or 680–750 nm (Chl autofluorescence) was used for detection. The experiments were repeated at least 5 times for every construct.

P-eIF2α Western blots

10-days-old seedlings grown on MS media were used for these experiments. Seedlings were treated with 200mM glyphosate for 5 minutes, and collected after 6 hours of recovery in MS, or UV-C exposed for around 25 minutes (9000 energy x2 + 4500. Stratalinker 1800) and collected immediately. Protein was

extracted using the P-eIF2α extraction buffer described elsewhere (Zhang *et al*. 2003). 20µg of protein were loaded in a 10% SDS-PAGE gel and inmunoblotting was performed using Phospho-eIF2α (Ser51) antibody (Cell Signalling) at a 1:2000 dilution and a secondary ECL anti-rabbit IgG horseradish peroxidase-linked whole antibody (GE Healthcare) at a 1:10.000 dilution and visualized using a chemiluminescence system.

Microarray experiments.

Total RNA was extracted from 20-days-old Col-0 and *ila 3*. Transcriptome analysis was done using the Agilent Arabidopsis (V4) Gene Expression Agilent 4x44 Microarray, which contained 43803 probes (60-mer oligonucleotides) and was used in a two-color experimental design. Three biological replicas of a comparison *ila3* and Col-0 wild-type plants, were performed. Sample RNA (0.5 µg) was amplified and labeled with the Agilent Low Input Quick Amp Labeling Kit. Hybridization and slide washing were performed with the Gene Expression Hybridization Kit and Gene Expression Wash Buffers, respectively. Slides were scanned in a GenePix 4000B microarray scanner, at 5 µm resolution. Image files were analyzed with Feature Extraction 9.5.1. Interarray analyses were performed with GeneSpring 11.5. Only those feautures for which the 'IsWellAboveBG' parameter was 1 in at least two out of three replicates was selected. To identify significantly expressed genes, a one-class significant analysis of microarrays (SAM) test (Tusher *et al.* 2001) was performed with adjustment according to Benjamini and Hochberg's method. Features were selected only if q value was below 1 after correction for multiple testing and expression ratio was greater than twofold different, for those genes having a valid value in the three replicates. Gene Set Enrichment analysis on Gene Ontology tools was performed using a logistic model based algorithm (Alonso *et al*. 2015) and a representative subset of the enriched GO-terms was obtained using a clustering algorithm (ReviGO) that relies on semantic similarity measures (Supek *et al*. 2011).
These microarrays data have been included in the GEO Omnibus database with the reference number GSE93312.

β-galactosidase assay

The C-terminal part of the ILA gene (ILA C-term), comprising amino acids 2098-2696, was fused to the hemagglutinin tag (HA) in the pAG425 vector (Alberti *et al*. 2007), under the control of the galactose inducible GAL promoter. W303 yeast cells harboring the p180 plasmid were transformed with the

pAG425 empty vector as a control, and the pAG425-ILA(C-term) construct. The p180 plasmid expressing a GCN4-LacZ fusion including the entire GCN4 5′-non-coding region with four upstream open reading frames inserted into YCp50, a low copy-number plasmid marked with URA3, has been described in (Yang *et al*. 2000).

Yeast strains were grown as follows: 20 mL of a saturated culture (grown in SD + glucose + 1% rafinose + dropout mix (arg, lys, met, ile, ser, thr, tyr, val, phe, inositol, p-aminobenzoic acid and adenine) were inoculated into 100 ml of SD + galactose + 1% rafinose + dropout and grown overnight at 30ºC. OD was measured and adjusted to 0,2 with control (SD + galactose + 1% rafinose + dropout mix) or stress medium (SD + galactose + 1% rafinose without dropout mix) . Yeasts were grown to an OD of 0.6, pelleted and maintained at -80ºC. Pellets were resuspended in 110µL of GTED (20% glycerol (v/v), 10mM Tris pH 7.6, 1mM EDTA pH8, 1mM DTT). 10µL of the mixture were used to measure the OD600 before the assay. Then 6mL of fresh TET solution (100µL toluene, 400µL ethanol, 50µL Triton X-100 20%, 50µL H_20) was added into each tube and cells were vortexed for membrane permeabilization. 5µL of cells were mixed with 95µL Z buffer (1M sodium phosphate, pH7, 10mM KCL, 1mM $MgSO_4$, 50mM β-mercaptoethanol. The reaction was started by adding 20µL of ONPG (4mg/ml o-nitrophenyl-beta-galactoside 13.3mM in 0.1 M sodium phosphate, pH7) into each tube (Time=0). Samples were incubated at 28°C until the yellow color developed and stopped by adding 50µL of 1M Na_2CO_3. Absorbance was monitored at 415nm. Arbitrary units of β-galactosidase activity = (680 x A415)/(time (m) x Vc(ml) x A660). Vc = 0.272 in this experiment.

Transmission electron microscopy (TEM)

Arabidopsis plants were grown on the greenhouse under long-day conditions for 30 days. For TEM, LR-white resin inclusion was performed fixing Arabidopsis leaves with glutaraldehyde 2.5%, washed three times (5 min each) with phosphate buffer 0.1M pH=7.2, and post-fixed with Osmium for 2h. After three washes with water (5 min each), they were sequentially dehydrated in ethanol 30%-90% and incubated for 2h in LR-white resin in ethanol 90%, LR-white resin in ethanol 100% and 100% LR-white resin. Ultrathin slides (60nm) were stained with 2% uranil acetate and plumb prior to viewing by transmission EM (TEM) using a Jeol JEM1010 microscope at 60kV. Images were acquired with a digital camera AMT RX80 (8Mpx).

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Conflict of Interest

None declared

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Table 1. **Photosynthetic parameters in Col-0. *ila3* and *gcn2-2* young and mature leaves.** Effect of leaf age on the photosynthetic rate ($A_{N;}$ µmol $m^{-2}s^{-1}$), stomatal conductance ($g_{s;}$ mol $m^{-2}s^{-1}$), substomatal CO_2 concentration ($C_{i;}$ mol mol^{-1}), transpiration rate (E; mmol $m^{-2}s^{-1}$), quantum efficiency of photosystem II (PhiPS2), minimal (F_o) and maximal (F_m) Chl a fluorescence in the dark adapted state, maximal photochemical efficiency (Fv/Fm) and SPAD index (a.u.) in wild-type (Col-0), *ila3* and *gcn2-2* plants. Each value is the mean of eight independent determinations in different plants. ANOVA was performed for comparisons among genotypes.

Leaf age	Genotype	A_N	g_s	C_i	E	PhiPS2	F_o	F_m	F_v/F_m	SPAD
Mature	Col-0	9.8	0.24 b	321	3.4 b	0.228	526	2847	0.814	22
	ila3	9.5	0.33 a	332	4.5 a	0.222	491	2627	0.809	21
	gcn2-2	9.6 NS	0.25 b	323 NS	3.5 b	0.219 NS	514 NS	2829 NS	0.817 NS	24 NS
Young	Col-0	8.1 a	0.18	290 a	2.3	0.210 a	550 a	2916 a	0.811 a	22 a
	ila3	4.8 b	0.20	342 b	2.7	0.185 b	460 b	2328 b	0.794 b	15 b
	gcn2-2	7.8 c	0.20 NS	316 a	2.8 NS	0.211 a	524 a	3007 a	0.825 a	23 a

For each leaf age, different letters indicate significant differences (P<0.05); NS: not significant

Figure 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 (SD gal – 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 (20ug) 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.

Figure 2. Root development is impaired in *ila* mutants. Representative roots of Col-0, *gcn2-2, ila3*, and two different lines of *ila3/gcn2-2* double mutant **(a)** as well as *ila2* **(b)** after growing vertically on MS medium during 20 days.

Figure 3. *Ila* mutants have defective chloroplast development. Transmission electron microscopy images of Col-0 **(a)**, *ila3* **(b)**, *gcn2-2* **(c)** and *ila2* **(d)** leaves chloroplasts showing internal thylakoid membranes. Plants were grown for 30 days in the greenhouse under long-day conditions. Scale bars: 800 nm **(A)** or 1 μm **(B,C,D)**.

Figure 4. *ila3/gcn2-2* double mutants maintain the chlorotic phenotype characteristic of the *ila3* mutant. (a) Rosette phenotype of plants grown in the greenhouse under long-day conditions for 20 days. **(b)** Histograms showing the photosynthetic rate (AN) and SPAD index in wild-type (Col-0), *gcn1, gcn2-2* and two *ila3/gcn2-2* lines. Each value is the mean of ten independent determinations in different plants. Results were subjected to an analysis of variance. The mean comparisons were performed with Tukey's test.

Figure 5. Representative GO categories enriched in *ila3* overexpressed and underexpressed genes. The scatterplot shows the cluster representatives (terms remaining after the redundancy reduction) in a two dimensional space derived by applying multidimensional scaling to a matrix of the GO terms' semantic similarities, according to REVIGO software. Color scale (log_{10} p-value).

Supplemental Figure 1: Bimolecular fluorescence complementation (BiFC) assay between AtGCN2 and the proteins indicated. Images were obtained from the YFP channel, chlorophyll channel, bright field and merged panel (see Materials and Methods). The following negative controls are included (AKINβ2- YFP^C /GCN2- YFP^N; GCN2- YFP^C / AKIN10- YFP^N; ILA-C-term- YFP^C /AKIN10- YFP^N. The following positive control is included: AKINβ2- YFP^C/ AKIN10- YFP^N. Autofluorescence is discarded for GCN2- YFP^C, ILA-C-term- YFP^C and GCN2- YFP^N.

Supplemental Figure 2. Western blot (HA antibody) showing the expression of ILA(C-term) in W303 yeast cells harboring the p180 plasmid transformed with the pAG425 empty vector or with pAG425-ILA(C-term). A band of approximately 70 kDa is observed, corresponding to the expected size of the ILA(C-term) protein fused to HA.

Supplemental Figure 3. Western blot assaying eIF2α phosphorylation on wild-type (Col0), and *ila2* seedlings, mock-treated and treated with UV-C to induce eIF2α phosphorylation. A differential band corresponding to P-eIF2α is shown (~54 kDa). Equal amount of protein (20ug) 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.

Supplemental Figure 4. Phenotype of representative Arabidopsis wild-type (Col-0) and *gcn2-2* mutant plants 20 **(a)** and 35 **(b)** days after sowing. Plants were grown in the greenhouse under long-day

conditions. **(c)** Western blot assaying eIF2α phosphorylation on wild-type (Col-0), *gcn2-2* and *gcn2-2/35S::GCN2* seedlings, mock-treated and treated with UV-C to induce eIF2α phosphorylation. A differential band corresponding to P-eIF2α is shown (arrow). Equal amount of protein (20ug) 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.

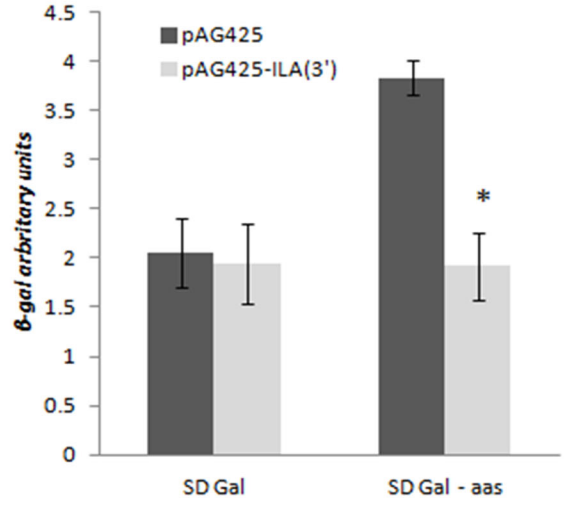
Supplemental Figure 5. *gcn2-2* mutant expresses a chimeric GCN2 mRNA, including the T-DNA. **(a)** Structure of the GCN2 gene and position of the T-DNA in the SALKseq_032196 insertion line. Exons (boxes) and introns (lines) are depicted approximately true to scale according to their lengths. Arrows indicate the position of the different primers used for RT-PCRs. **(b)** RT-PCRs for Col-0 and *gcn2-2* mutant line using primers flanking the T-DNA (left panel), after the T-DNA (middle panel) or including a region of the T-DNA (right panel). FP: Forward Primer. RP: Reverse Primer. **(c)** Panel showing the expected sized (genomic and cDNA) for the different RT-PCRs performed.

Supplemental Table 1. Differentially expressed genes in the transcriptomic experiment comparing wild-type and *ila3* seedlings. Columns correspond to: A: Agilent Code. B: AGI code. C: NCBI Gene ID. D: URL to NCBI. E: numerator after SAM test (average fold-change) F: q-value. G-I. Independent ratios ila3/wild-type in the three replicates. J: Description. Data are in log2 scale.

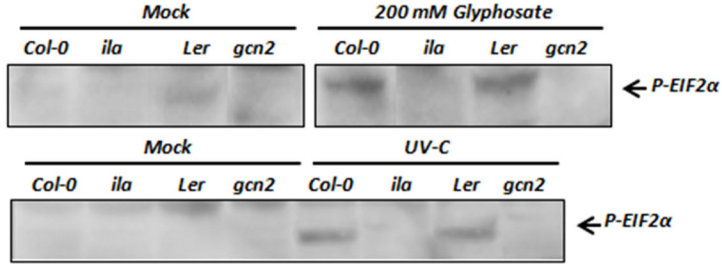
Supplemental Table 2. Gene ontology categories enriched among the genes differentially expressed in the transcriptomic experiment comparing wild-type and *ila3* seedlings, according to ReviGO software.

1

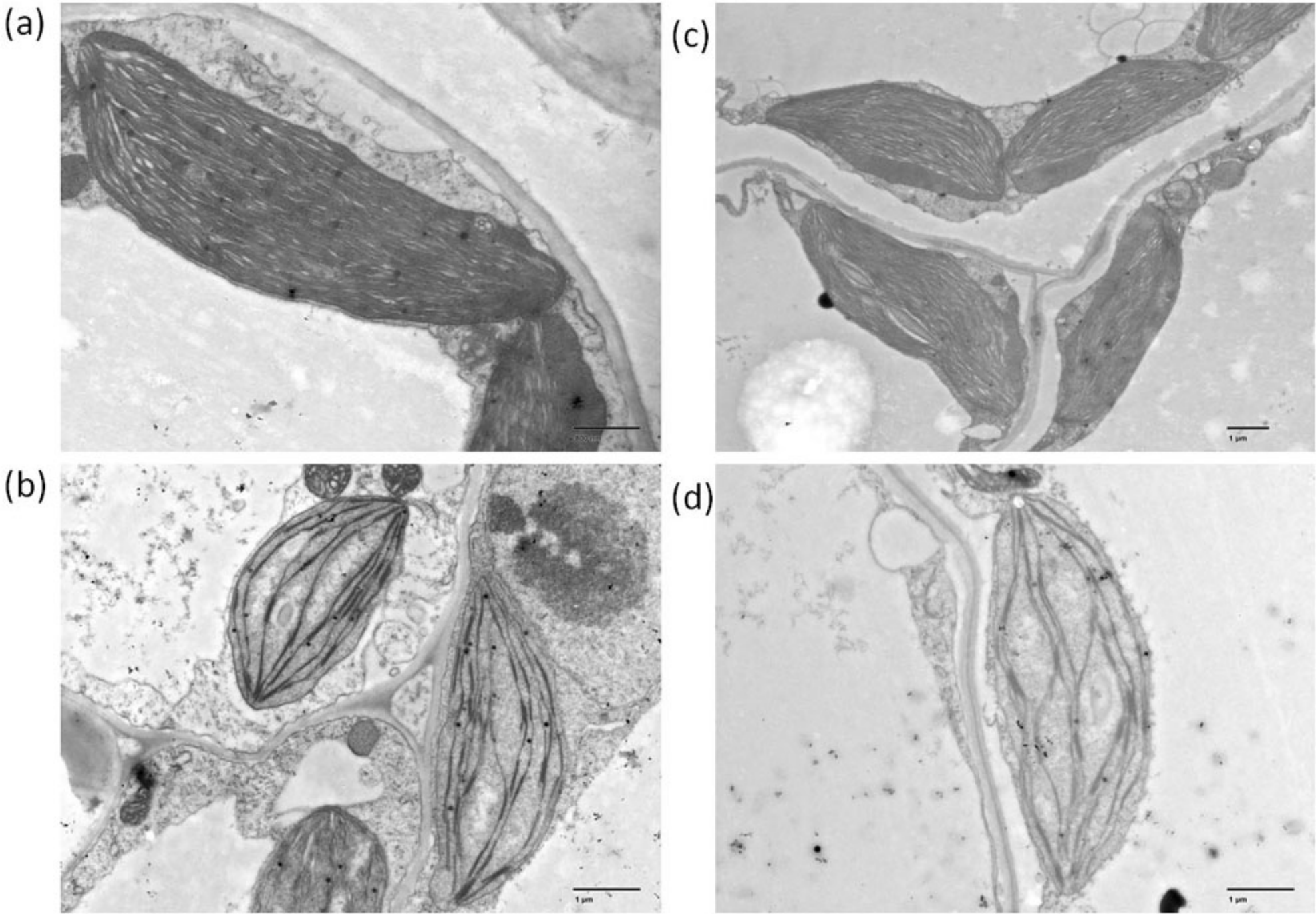
A

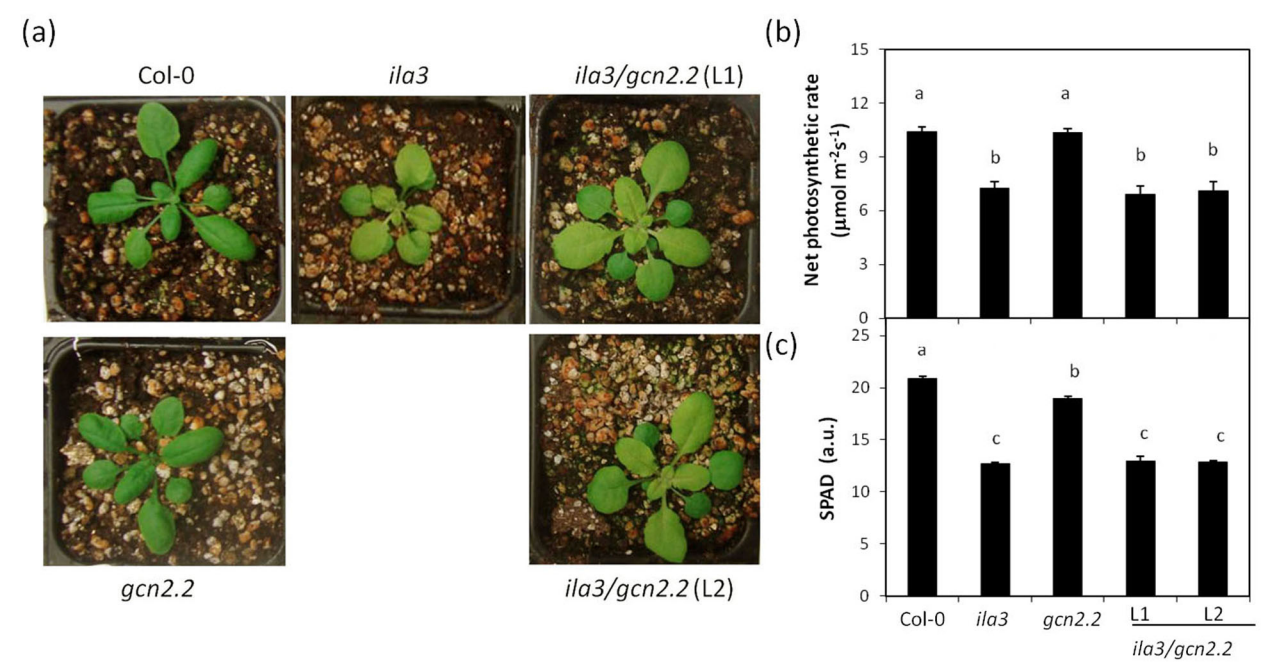

4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
pAG425
pAG425-ILA(3')
β-gal arbitrary units
*
SD Gal
SD Gal - aas

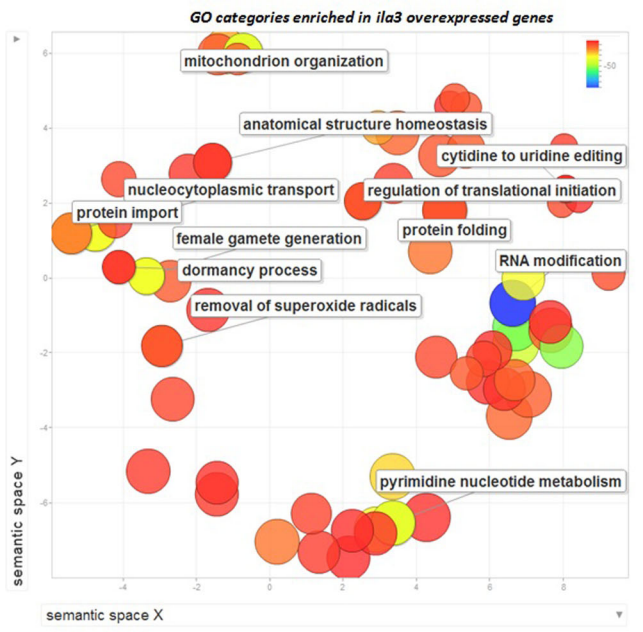
B

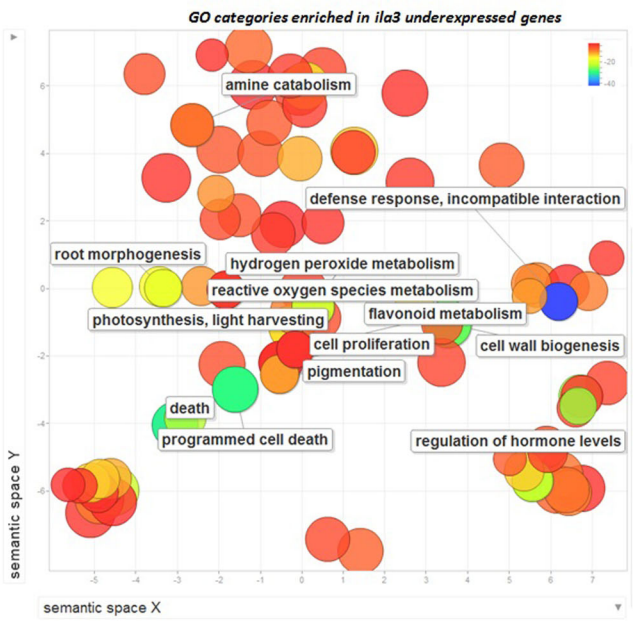

Mock
200 mM Glyphosate
Col-0
ila
Ler
gcn2
Col-0
ila
Ler
gcn2
P-EIF2α
Mock
UV-C
Col-0
ila
Ler
gcn2
Col-0
ila
Ler
gcn2
P-EIF2α

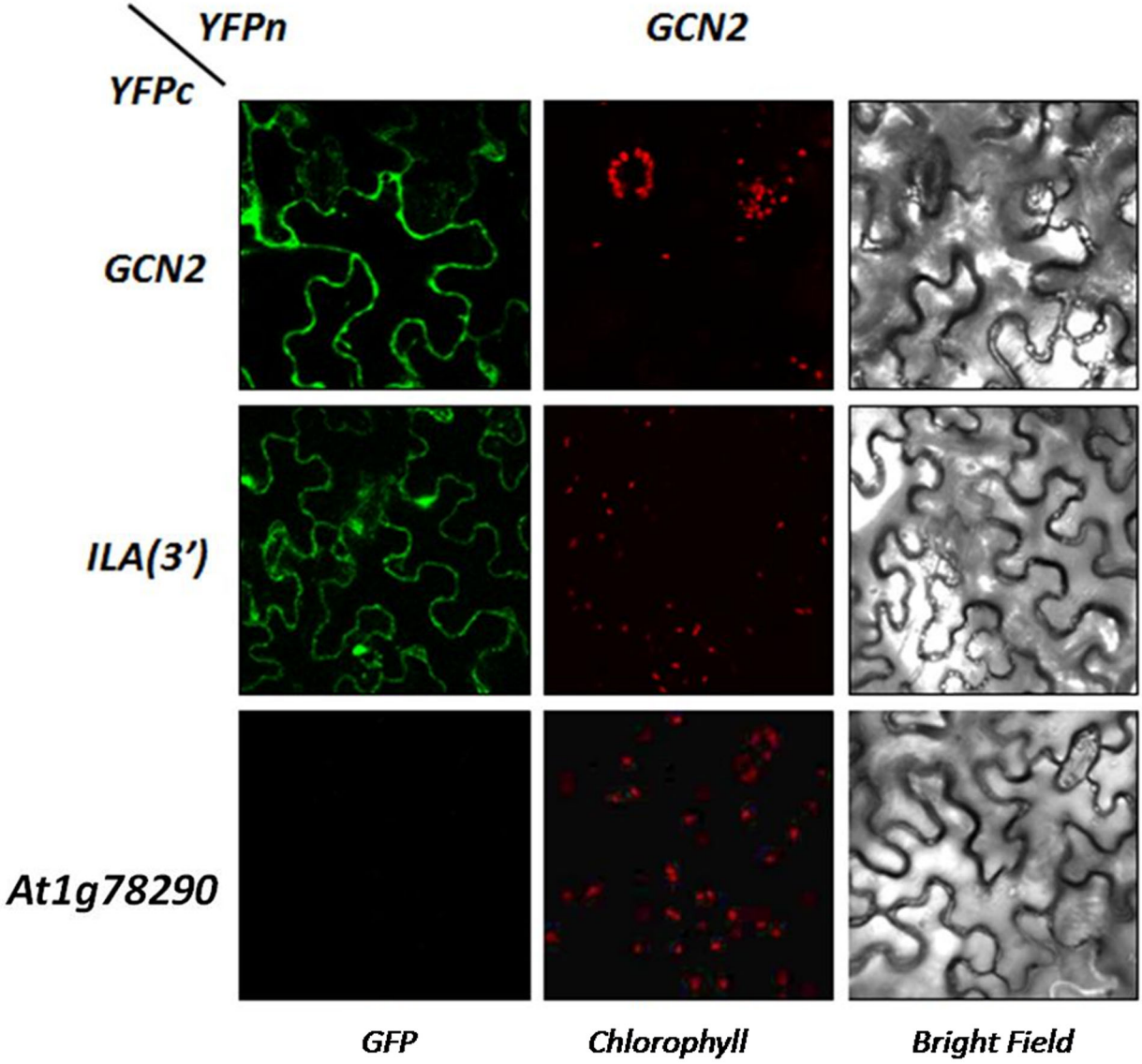

ila3/gcn2.2
Col-0
gcn2.2
ila3
L1
L2
(a)
(b)
Col-0
ila2


(a)
(c)
(b)
(d)
1 µm
1 µm
1 µm


(a)
Col-0
ila3
ila3/gcn2.2 (L1)
gcn2.2
ila3/gcn2.2 (L2)
(b)
Net photosynthetic rate (μmol m-2s-1)
15
12
9
6
3
0
a
b
a
b
b
(c)
SPAD (a.u.)
25
20
15
10
5
0
a
c
b
c
c
Col-0
ila3
gcn2.2
L1
L2
ila3/gcn2.2


GO categories enriched in ila3 overexpressed genes
mitochondrion organization
anatomical structure homeostasis
cytidine to uridine editing
nucleocytoplasmic transport
regulation of translational initiation
protein import
female gamete generation
protein folding
dormancy process
RNA modification
removal of superoxide radicals
pyrimidine nucleotide metabolism
-50
semantic space Y
semantic space X


GO categories enriched in ila3 underexpressed genes
amine catabolism
defense response, incompatible interaction
root morphogenesis
hydrogen peroxide metabolism
reactive oxygen species metabolism
photosynthesis, light harvesting
flavonoid metabolism
cell proliferation
cell wall biogenesis
pigmentation
death
programmed cell death
regulation of hormone levels
-20
-40
semantic space Y
semantic space X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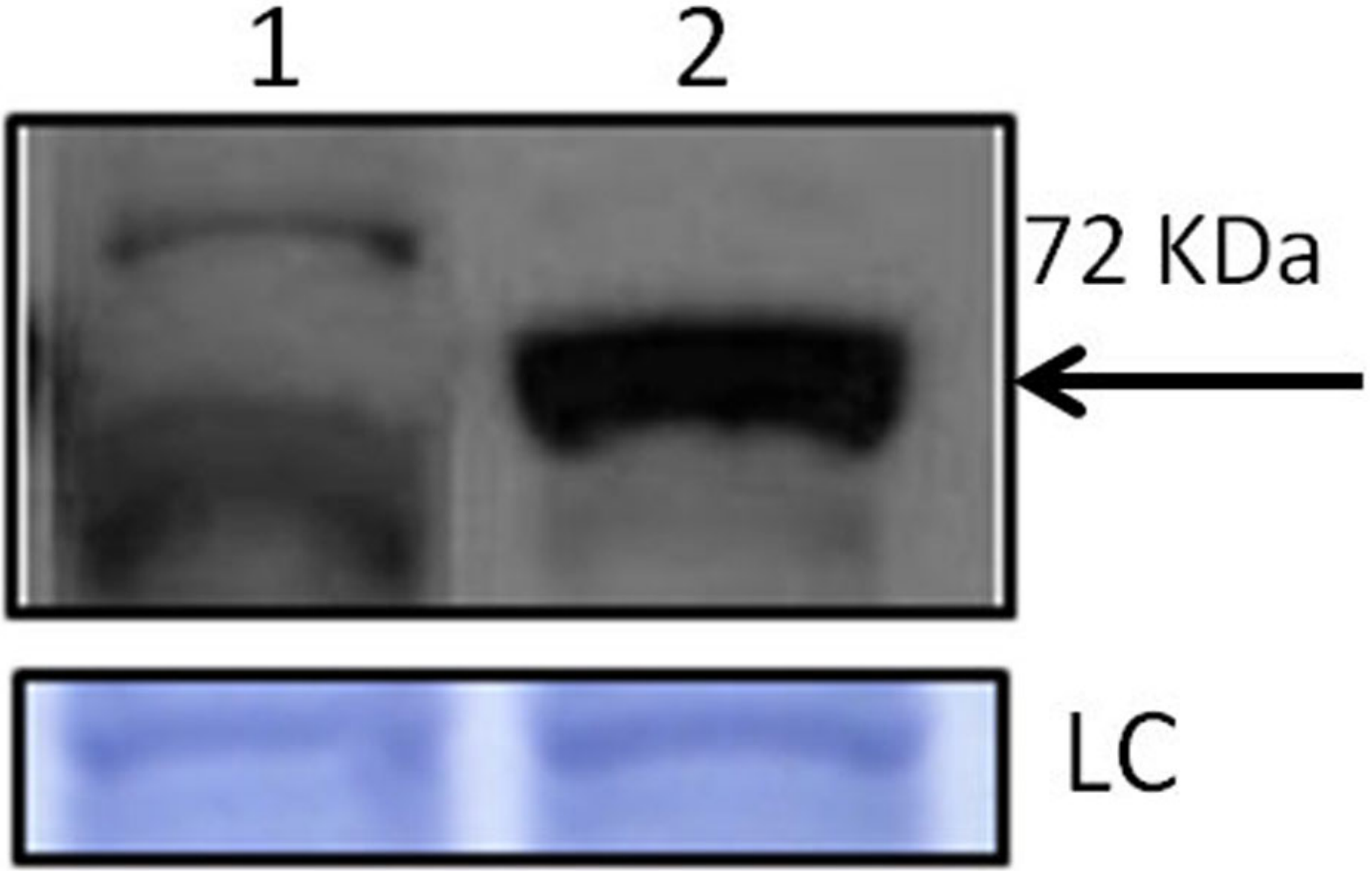
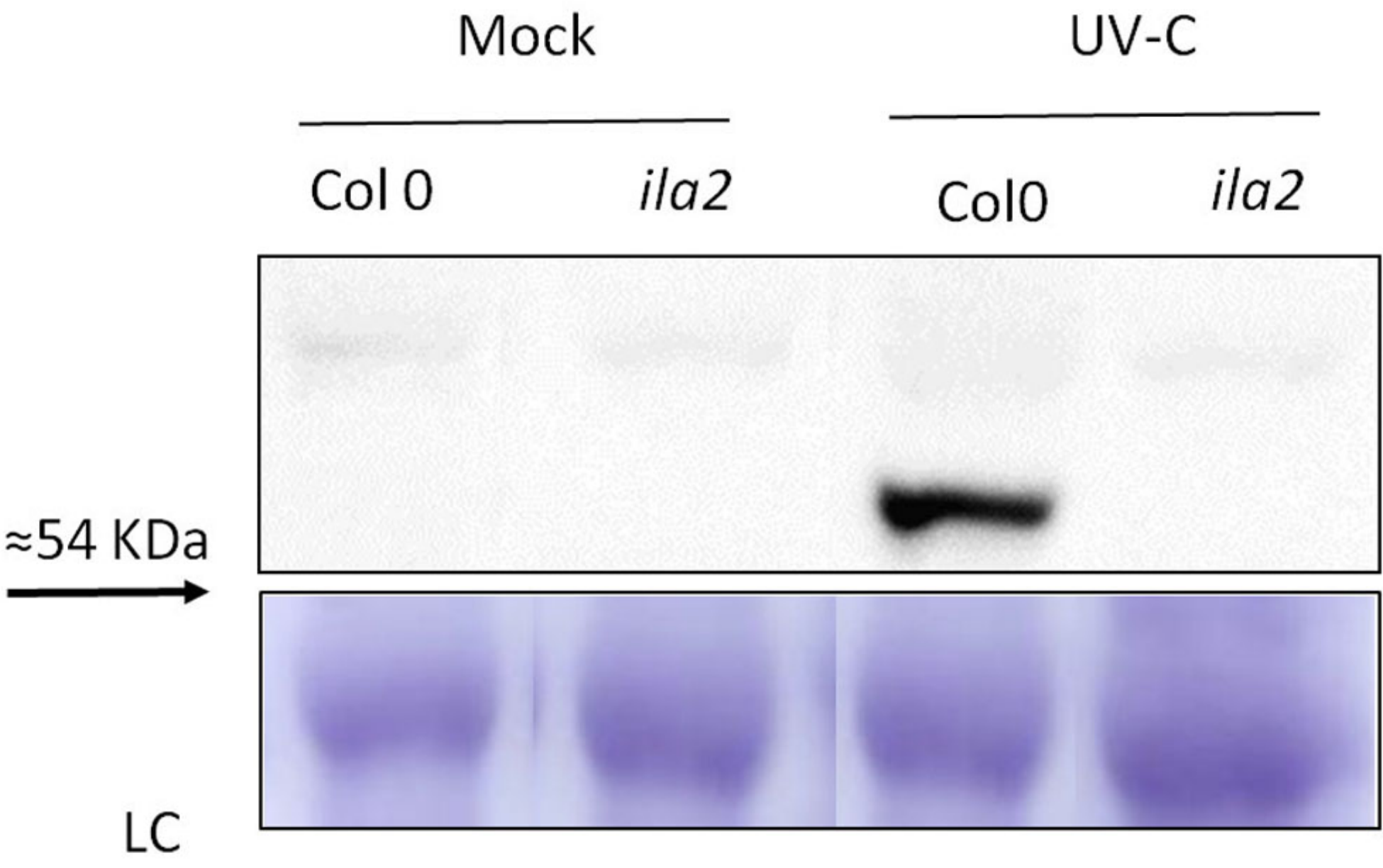
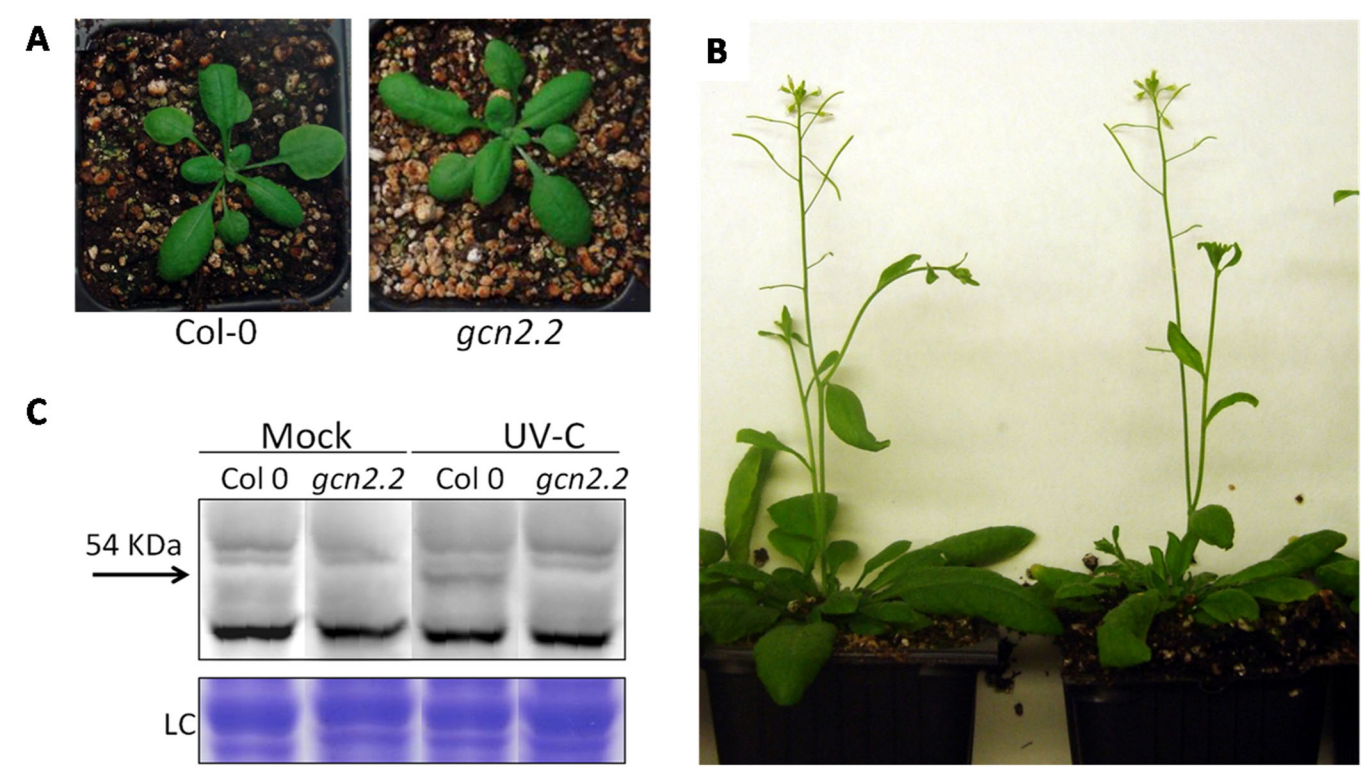
YFPc
GCN2
GCN2
ILA(3')
At1g78290
GFP
Chlorophyll
Bright Field


1
2
72 KDa
LC


Mock
UV-C
Col 0
ila2
Col0
ila2
≈54 KDa
LC


A
Col-0
gcn2.2
B
C
Mock
UV-C
Col 0
gcn2.2
Col 0
gcn2.2
54 KDa
LC