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Caracterización del sistema GCN en plantas mediante la utilización de mutantes de pérdida de función

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A mi “Abu” Maribel,
Te fuiste cuando empezaba lo mejor.

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Resumen:

La proteína quinasa GCN2 es una proteína conservada en todos los eucariotas implicada en el control de la traducción en condiciones de estrés. Está considerada un punto clave en el control de la homeostasis celular y un sensor de distintas condiciones de estrés. El estrés que inició su caracterización en levaduras y células animales es el ayuno de aminoácidos, pero recientemente se ha observado activación de este sistema ante multitud de estreses tanto bióticos como abióticos. El sistema GCN se ha descrito ampliamente en *Saccharomyces cerevisiae*: GCN2 se une a las proteínas GCN1 y GCN20, permitiendo la activación de la quinasa en situaciones de ayuno de aminoácidos. GCN2 se activa por tRNA no cargados, y posteriormente fosforila al factor de traducción eIF2α, lo que conlleva una reducción de la síntesis global de proteínas, pero también una mayor traducción de mRNA específicos, como los que codifican a GCN4. Este factor de transcripción regulará la expresión de nuevos genes, lo que permite que la célula pueda iniciar una respuesta de adaptación al estrés.

En plantas se desconoce con detalle como el sistema GCN contribuye a mitigar el estrés y controlar la homeostasis. Las tres proteínas conocidas de este sistema tienen homólogos en *Arabidopsis*. Diversos estudios indican que el mecanismo de actuación de GCN2 en plantas presenta muchas incógnitas. Mientras que la quinasa GCN2 de plantas se activa bajo diferentes situaciones de estrés, la participación de los homólogos de GCN1 y GCN20 en estos procesos es controvertida, y recientemente se ha propuesto un nuevo papel para GCN1 en la traducción, independiente de GCN2.

El homólogo de GCN1 en plantas está implicado en la inmunidad innata y adquirida y sus líneas mutantes presentan fenotipos muy diferentes a los de las líneas mutantes en GCN2. La relación funcional entre estos dos genes sigue siendo difícil de definir en plantas. En esta tesis, demostramos que, aunque los genes GCN1 y GCN2 de *Arabidopsis* son necesarios para mediar la fosforilación de eIF2α tras tratamientos con glifosato, inhibidor de la biosíntesis de aminoácidos aromáticos, los mutantes de pérdida de función de ambas líneas desarrollan distintos fenotipos de raíz y cloroplasto. Los experimentos de microscopía electrónica revelan que los mutantes en GCN1, pero no en GCN2, se ven afectados en la biogénesis de cloroplastos, lo que explica el fenotipo macroscópico observado previamente para estos mutantes. Los mutantes en GCN1 presentan una compleja reprogramación transcripcional que afecta, entre otros, a las respuestas relacionadas con los mecanismos de defensa, fotosíntesis y al correcto plegamiento de las proteínas.

Los análisis de los dobles mutantes sugieren que GCN1 en plantas tiene otra función, que es independiente de la fosforilación de GCN2 y eIF2α. Estos resultados indican que estos dos genes tienen funciones comunes, pero también distintas, en *Arabidopsis*.

Por otro lado, mostramos que ninguno de los cinco genes homólogos a GCN20 en *Arabidopsis* en necesario para la fosforilación de eIF2α. Además, los fenotipos bajo estrés abiótico de plantas mutantes en los mismos, y el desarrollo de sus cloroplastos, sugiere que GCN20 está funcionalmente relacionado con GCN1, pero no con GCN2, algo que se confirma ya que los mutantes *gcn1* y *gcn20* comparten una reprogramación transcripcional similar, afectando a la fotosíntesis y a las respuestas frente al estrés.

Identificamos la proteína quinasa GCN2 como un componente celular que fomenta la acción del glifosato en *Arabidopsis*. Los estudios comparativos que utilizan plántulas mutantes de pérdida de función de GCN2 muestran que el programa molecular que la planta despliega después del tratamiento con el herbicida no está teniendo lugar. Además, las plantas adultas *gcn2* muestran una menor inhibición de la fotosíntesis, y acumulan menos ácido siquímico que las de tipo silvestre después del tratamiento con glifosato. Algo similar ocurre tras el tratamiento con luz ultravioleta UV-B, donde mutantes de pérdida de función son más resistentes. La activación de GCN2 ante este estrés es independiente del fotorreceptor UV-B (UVR8) y de sus componentes de señalización aguas abajo y de la vía de señalización de estrés de las MAP quinasas.

Resum:

La proteína quinasa GCN2 és una proteïna conservada en tots els organismes eucariotes implicada en el control de la traducció en condicions d'estrés. Està considerada un punt clau en el control de l'homeòstasis cel.lular i es un sensor de diferents i variades condicions d'estrés. L' estrés que va iniciar la seua caracterització en llevats i cèl.lules animals és el dejuni d' aminoàcids, però recentment s'ha observat l'activació d'aquest sistema davant de multitud d'estressos tant biòtics com abiòtics. El sistema GCN ha segut descrit ampliament en *Saccharomyces cerevisiae*: GCN2 s'uneix a les proteïnes GCN1 y GCN20, permitint l'activació de la quinasa en situacions de dejuni d'aminoàcids. GCN2 s'activa per tRNA no carregats, i posteriorment fosforila el factor de traducció eIF2α, donant lloc a una reducció de la síntesi global de proteïnes, però també una major traducció de mRNA específics, com els que codifiquen a GCN4. Aquest factor de transcripció regularà l'expressió de nous gens, el que permet que la cèl·lula puga iniciar una resposta d'adaptació a l'estrés.

En plantes es desconeix amb detall com el sistema GCN contribueix a mitigar l'estrés i controlar l'homeòstasi. Les tres proteïnes conegudes d'aquest sistema tenen homòlegs en *Arabidopsis*. Diversos estudis indiquen que el mecanismo d'actuació de GCN2 en plantes presenta moltes incògnites. Mentres que la quinasa GCN2 de plantes es activada en diferents situacions d'estrés, la participació dels homòlegs de GCN1 i GCN20 en aquests processos és controvertida, i recentment s'ha proposat un nou paper per a GCN1 en la traducció, independent de GCN2.

L'homòleg de GCN1 en plantes està implicat en la inmunidad innata i adquirida i les seues línies mutants presenten fenotips molt diferents als de les línies mutants en GCN2. La relació funcional entre estos dos gens continua sent difícil de definir en plantes. En esta tesi, demostrem que, encara que els gens GCN1 i GCN2 d' *Arabidopsis* són necessaris per a donar lloc a la fosforilació d'eIF2α després de ser tractada amb glifosato, inhibidor de la biosíntesi d' aminoàcids aromàtics, els mutants de pèrdua de funció d'ambes línies desenvolupen distints fenotips d'arrel i cloroplast. Els experiments de microscòpia electrònica revelen que els mutants en GCN1, però no en GCN2, es veuen afectats en la biogènesis de cloroplastos, el que explica el fenotip macroscòpic observat prèviament per a estos mutants. Els mutants en GCN1 presenten una complexa reprogramació transcripcional que afecta, entre d'altres, a les respostes relacionadaes amb els mecanismes de defensa, fotosíntesi i al correcte plegament de les proteïnes. Els análisis dels dobles

mutants sugereixen que GCN1 en plantes té una altra funció, que és independent de la fosforilació de GCN2 i eIF2α. Aquests resultats indiquen que estos dos gens tenen funcions comuns, pero també diferentes, en *Arabidopsis*.

D'altra banda, demostrem que ningun dels cinc gens homòlegs a GCN20 en *Arabidopsis* és necessari per a la fosforilació d' eIF2α. Ademés, els fenotips baix estrés abiòtic de plantes mutants en ells mateix, i el desenvolupament dels seus cloroplasts, sugereixen que GCN20 està funcionalment relacionat amb GCN1, però no amb GCN2, cosa que es confirma ja que els mutants *gcn1* i *gcn20* compartixen una reprogramació transcripcional similar, afectant a la fotosíntesi i les respostes davant l'estrés.

Identifiquem la proteïna quinasa GCN2 com un component cel.lular que fomenta l'acció del glifosato en *Arabidopsis*. Els estudis comparatius que utilitzen plántules mutants de pèrdua de funció de GCN2 mostren que el programa mol.lecular que la planta desplega després del tractament amb herbicida no está ocorreguent. Ademés, les plantes adultes *gcn2* presenten una menor inhibició de la fotosíntesi, i acumulen menys àcid siquímic que les de tipus silvestre després de ser tractades amb glifosato. S'obté un resultat semblant després del tractament amb llum ultravioleta UV-B, on els mutants de pèrdua funció són més resistents. L'activació de GCN2 davant d'aquest estrés és independent del fotorreceptor UV-B (UVR8) i dels seus components de senyalització aigües avall i de la via de senyalització d'estrés de les Map quinases.

Abstract:

The GCN2 protein kinase is a conserved protein in all eukaryotes involved in translation control under stress conditions. It is considered a key point in the control of cellular homeostasis and a sensor for a wide variety of stress conditions. Aminoacid fasting was the stress that started its characterization in yeast and animal, but recently activation of this system has been observed in both biotic and abiotic stresses. The GCN system has been extensively described in Saccharomyces cerevisiae: GCN2 binds to GCN1 and GCN20 proteins, allowing kinase activation in aminoacid fasting situations. GCN2 is activated by uncharged tRNAs, and subsequently phosphorylates the translation factor eIF2α, leading to a reduction in overall protein synthesis, but also a greater translation of specific mRNAs, such as those encoding GCN4. This transcription factor will regulate the expression of new genes, allowing the cell to initiate an adaptive response to stress.

In plants, it is not deeply known how the GCN system helps to alleviate stress and control homeostasis. All three known proteins in this system have homologs in *Arabidopsis*. Some studies indicate that the mechanism of action of GCN2 in plants presents many gaps. While plant GCN2 kinase is activated under different stress situations, the involvement of GCN1 and GCN20 homologs in these processes is controversial, and recently it has been proposed a new role for GCN1 in translation, independent from GCN2.

The GCN1 homolog in plants is involved in innate and acquired immunity and its mutant lines present very different phenotypes from those of the GCN2 mutant lines. The functional relationship between these two genes is difficult to define in plants. In this thesis, we prove that, although the *Arabidopsis* GCN1 and GCN2 genes are necessary to mediate in the phosphorylation of eIF2α after treatments with glyphosate, an inhibitor of aromatic aminoacid biosynthesis, the loss of function mutants of both lines develop different phenotypes of root and chloroplast. Electron microscopy experiments reveal that the mutants in GCN1, but not in GCN2, are affected in chloroplast biogenesis, which explains the macroscopic phenotype previously observed for these mutants. The mutants in GCN1 present a complex transcriptional reprogramming that affects, among others, the responses related to defense mechanisms, photosynthesis and the correct folding of proteins. Analysis of the double mutants suggests that GCN1 in plants has another function, which is independent from phosphorylation of GCN2 and eIF2α. These results show that both genes have common and different functions in *Arabidopsis*.

On the other hand, we show that none of the five GCN20 homologous genes in *Arabidopsis* is necessary for the phosphorylation of eIF2α. Furthermore, the phenotypes under abiotic stress of mutant plants in them, and the development of their chloroplasts, suggest that GCN20 is functionally related to GCN1, but not to GCN2, which is confirmed because the *gcn1* and *gcn20* mutants share a similar transcriptional reprogramming and affects photosynthesis and stress responses.

We identify the GCN2 protein kinase as a cellular component that promotes the action of glyphosate in *Arabidopsis*. Comparative studies using GCN2 loss-of-function mutant seedlings show that the molecular program that the plant develops after the treatment with the herbicide is not taking place. Furthermore, adult *gcn2* plants show less inhibition of photosynthesis, and accumulate less shikimic acid than wild-type ones after glyphosate treatment. Something similar happens after treatment with UV-B ultraviolet light, where loss-of-function mutants are more resistant. Activation of GCN2 in the face of this stress is independent of the UV-B photoreceptor (UVR8) and its downstream signaling components and the stress signaling pathway of MAP kinases.

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Abreviaturas:

ABC: ATP-Binding Casette

aRNA: Aminoallyl labeled Ribonucleic Acid

AtGCN: *Arabidopsis* General Control Non-derrepresive

ATF4: Factor activador de la transcripción

ATP: Trifosfato de adenosina/Adenosín trifosfato

ATR: Ataxia telangiectasia-mutated and Rad3-related

CDK: Cyclin-Dependent Kinase

CDP: Dímeros de pirimidina

CTD: Dominio de dimerización C-Terminal

cDNA: Ácido Desoxirribonucleico Complementario

Col-0: Ecotipo Columbia de *Arabidopsis thaliana*

DNA: Ácido Desoxirribonucleico

EF3: Factor de elongación de la traducción 3

eIF: Factores eucarióticos de iniciación de la traducción

eIF2B: Factores eucarióticos de iniciación de la traducción 2 B

eIF2α: Subunidad alfa del factor de iniciación de la traducción

GAAC: Control General de los Aminoácidos

GCN: General Control Non-derrepresive

GTP/GDP: Guanosin trifosfato/Trifosfato de guanosina

HisRS: Histidil-tRNA sintetasas

HRI: Inhibidor regulado por hemina

ISR: Respuesta al estrés integrada

JA: Ácido jasmónico

Ler: Ecotipo *Landsberg* de *Arabidopsis thaliana*

MAPK/AMPK: Proteína quinasa activada por mitógeno

MMS: Metil Metano sulfonato

mRNA: Ácido Ribonucleico Mensajero

PCR: Reacción en Cadena de la Polimerasa

PERK: Proteína quinasa regulada por estés en el retículo endoplásmatico.

PI3P: Phosphatidylinositol 3-phosphate (fosfatidil inositol-3-fosfato)

PK: Protein kinase/ Proteina quinasa

PKR: Proteína quinasa regalada por el RNA de doble cadena

RNA: Ácido Ribonucleico

rRNA: Ácido Ribonucleico Ribosómico

ROS: Especie reactiva de oxigeno

SCORD: *Susceptive to coronatone (COR)-Deficient*

Ser: Serina

SnRK: Snf1-Related Kinases

TAIR: The *Arabidopsis* Information Resource

TC: Complejo ternario

TOR: Target Of Rapamycin (Diana de rapamicina)

TORC1: TOR Complex 1 (Complejo TOR1)

TORC2: TOR Complex 2 (Complejo TOR2)

Trp: Triptófano

tRNA: Ácido Ribonucleico Transferente

uORF: Upstream Open Reading Frames

UV: Ultravioleta

UVRB: Radiación Ultravioleta/ B

UVR8: Resistencia ultravioleta Locus 8

1.INTRODUCCIÓN

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1.1. Estrés celular y mecanismos generales de control

1.1.1. Estrés ambiental o abiótico e impacto sobre las plantas

Las plantas, al igual que cualquier ser vivo en su medio natural, están expuestas a todo tipo de estreses, tanto bióticos como abióticos, y han desarrollado mecanismos de control basados en la reprogramación de toda su expresión génica, de forma que, mediante diferentes puntos de regulación, y mediante cascadas de factores de transcripción, activan o reprimen una serie de genes implicados en la respuesta a los diferentes estímulos. La mayoría de los estreses presentan un impacto negativo sobre el crecimiento de la planta, afectando a su balance energético de forma importante, ya que la planta utiliza todas sus reservas energéticas disponibles para combatir dichos estreses. Ese impacto negativo en común, es el responsable de que las respuestas sean convergentes y sus puntos de regulación y patrones de respuesta estén en muchos casos superpuestos (Baena-González et al., 2008).

Los estreses se clasifican en bióticos (biológicos) o abióticos (no biológicos). Entre los factores de estrés abiótico, los más importantes son la sequía, la salinidad y las altas temperaturas; aunque otros como la radiación ultravioleta B (UVRB), las inundaciones y la polución están ganando relevancia por su impacto actual en los cultivos. (Rosa et al., 2009). Los procesos biológicos que se ven afectados en las plantas a causa de estos estreses son: el crecimiento celular, la fotosíntesis, la distribución del carbono, el metabolismo de los lípidos e hidratos de carbono, la homeostasis osmótica, la síntesis de proteínas y la expresión génica. (Rosa et al., 2009). Sus efectos pueden ser generales o específicos, al igual que los mecanismos de respuesta; aunque se dan patrones específicos de adaptación en cada tipo de estrés, existe una respuesta general a cada uno de ellos. En la naturaleza, las plantas se ven sometidas continuamente a uno u otro tipo de estrés, o a una combinación de varios de ellos, por los que los estudios enfocados en estas respuestas generales son muy importantes si se quiere determinar los patrones de adaptación de las plantas ante cualquier condición de estrés.

La habilidad o capacidad de las plantas para tolerar los estreses es un factor decisivo que determina la calidad y rendimiento de los cultivos (Hey et al., 2010). Los estreses

abióticos son la causa de la reducción de hasta un 50% de la producción de los cultivos alimentarios. La homeostasis de las plantas se ve constantemente amenazada por la incapacidad de poder escapar de las fluctuaciones ambientales. Estudios recientes postulan que probablemente las señales que percibe la planta al inicio de las situaciones de estrés proceden de diferentes condiciones que generan unas respuestas similares compartidas, de forma que la planta pueda empezar a defenderse antes de elaborar una respuesta específica. De ahí la importancia de tener una señal de energía compartida para activar la respuesta transcripcional frente a varios tipos de estrés. La deficiencia de energía está relacionada con la mayoría de las perturbaciones ambientes, ya que su impacto afecta directa o indirectamente a la fotosíntesis y/o a la respiración de las plantas (Baena-González et al., 2009). Por ello, conocer los mecanismos de defensa a estos estreses es fundamental para diseñar estrategias que ayuden a mejorar los rendimientos de los cultivos y la producción de los mismos.

1.1.2. Estrés por ayuno de nutrientes

El estrés por ayuno de nutrientes se define como: “el conjunto de efectos adversos para el ser vivo que provocan una disminución en la disponibilidad de los componentes moleculares necesarios para el mantenimiento y desarrollo de la célula”. Esta falta de nutrientes provoca un desajuste energético de la célula, impidiendo un correcto funcionamiento de los procesos esenciales tales como la síntesis de biomoléculas, implicadas en el crecimiento y división celular (Lindsley et al. 2004).

Todos los organismos tienen la capacidad de detectar la disponibilidad de nutrientes y ajustar el flujo entre las rutas metabólicas según las necesidades del momento (Lindsley et al. 2004), aunque cada uno de ellos lo afronte de formas distintas. Los organismos unicelulares se relacionan con el medio de forma directa, de manera que los compuestos que los rodean actúan tanto de señalizadores como de nutrientes. (Wilson et al., 2002). Por otro lado, en los organismos pluricelulares superiores como las plantas, entran en juego factores extracelulares como las hormonas, además de la adaptación de cada célula a su propio microambiente. La respuesta celular general al estrés por ayuno de nutrientes se realiza en dos fases. La primera fase se basa en la percepción del estímulo a través de sensores y la segunda fase en la reprogramación de la expresión génica, de forma que se movilizan represores y activadores transcripcionales. Los represores reconocen secuencias específicas de DNA a las que se unen reprimiendo la expresión génica,

y los activadores transcripcionales reclutan la maquinaria transcripcional necesaria para expresar los genes cuyos promotores recaen bajo su control. (Natarajan et al., 2002).

1.1.3. Respuesta a estrés por ayuno de nutrientes en plantas

El estrés por ayuno de nutrientes en plantas se traduce en un déficit de energía, por tanto, la respuesta al mismo es igual a otras situaciones que producen privación de energía, como por ejemplo la disminución de horas de luz. (Baena-Gonzáles et al., 2008). El ayuno, que suele ser mayoritariamente de carbono y/o nitrógeno, puede ser causado por el secuestro de moléculas de carbono por parte de algún patógeno o por factores abióticos como las inundaciones, las sequías, las temperaturas extremas, la salinidad excesiva, el agotamiento del suelo o la polución. Todos estos factores interfieren en la captación, asimilación y procesamiento de los nutrientes por parte de la planta (Baena-González et al., 2007), de forma que se desencadenan una serie de respuestas coordinadas para combatir dichos problemas.

Los procesos de estrés por ayuno de nutrientes se encuentran muy bien descritos en levaduras, organismo modelo empleado para estudiar mecanismos conservados a lo largo de la evolución. Pero en el caso de las plantas, como *Arabidopsis thaliana*, pese a haberse encontrado homólogos de casi todos los genes implicados, sigue siendo un misterio como las rutas se articulan y se coordinan entre si para regular y adaptarse a dichos estreses. Las tres rutas más importantes implicadas en la respuesta a estrés son: la ruta TOR, la ruta SnRK1 y la ruta GCN2 o de control general de los aminoácidos (GAAC).

1.1.3.1. TOR

Una de las rutas de detección intracelular de nutrientes mejor conservadas en eucariotas a lo largo de la evolución es la que implica a TOR (*Target of Rapamycin*) o diana de la rapamicina. Es una Serina/Treonina quinasa perteneciente a la familia de las fosfatidilinositol-quinasas, una familia altamente conservada desde levaduras a mamíferos. TOR es importante para las células ya que funciona como un regulador central del crecimiento celular, si se inhibe disminuye el crecimiento y se produce arresto de la fase G1 (Harris and Lawrence, 2003). A su vez, esta quinasa está regulada por una amplia variedad de señales que incluyen factores de crecimiento, nutrientes y condiciones de estrés. Fue descubierta en *S. cerevisiae* mientras se realizaban rastreos de mutaciones que conferían resistencia al antifúngico rapamicina (Heitman et al.,

1991). La presencia de este antifúngico inhibe la función de TOR y produce un fenotipo idéntico al del ayuno de nutrientes (Barbet et al., 1996).

Tras la identificación original de TOR en levadura S. cerevisiae, TOR también fue identificado en mamíferos, gusanos y plantas, sugiriendo que se trata de una proteína conservada en todas las formas de vida eucariota tanto a nivel estructural como por ser diana del complejo FKBP-rapamicina (Schmelzle and Hall et al., 2000)

En levadura, TOR está compuesto por dos proteínas, TOR1 y TOR2, mientras que en el resto de eucariotas, incluyendo Arabidopsis, esta función es realizada por una sola proteína. En el caso de S. cerevisiae, ambos proteínas juegan un papel central en el control del crecimiento celular como parte de una ruta separada en dos ramas de señalización. Aunque son estructuralmente similares, TOR1 y TOR2 no son funcionalmente idénticas. (Helliwel et al., 1994; Kunz et al., 1993). TOR2 tiene una función redundante con TOR1 y otra función única (Hall, 1996; Helliwel et al., 1998)

La rama de señalización común de la ruta TOR se compone de varias vías efectivas que controlan una amplia variedad de parámetros que en conjunto determinan la masa celular, se considera que controla el crecimiento celular en el tiempo. Mientras que la rama mediada unidamente por TOR2 controla espacialmente el crecimiento celular. De esta forma estas dos ramas separadas de la ruta TOR integran el control en el tiempo y en el espacio del crecimiento celular (Crespo and Hall, 2002; Loewith and Hall, 2011), promoviendo el anabolismo o el catabolismo en función de las condiciones ambientales en las que se encuentren la célula. Estas proteínas forman dos complejos, TORC1 y TORC2.

- **TORC1:** Es un complejo quinasa multiproteíco, es un sensor de varias señales celulares y ambientales que incluyen la abundancia y calidad de nutrientes, el estatus energético y las señales de crecimiento. Responde coordinando actividades asociadas con el crecimiento y la proliferación celular (Loewith and Hall, 2011). Todas estas señales convergen en el mismo tipo de respuesta: primero se inactiva TOR, y esto produce una disminución de la síntesis de proteínas, induciendo a la autofagia y salida del ciclo celular, llevando a la célula a la fase G0. Entre las señales que inhiben a TORC1 se encuentra la exposición a rapamicina y la disminución en el medio de los niveles de carbono, nitrógeno, fosfato y aminoácidos (Loewith et al., 2002) (Fig.1).

- **<u>TORC2</u>:** Regula la síntesis del citoesqueleto de actina, la endocitosis, la síntesis de esfingolípidos y la respuesta transcripcional al estrés. Las señales que posiblemente regulan el complejo de TORC2 son los ribosomas y el estrés en la membrana. En las células de mamífero y posiblemente también en las de levadura, TORC2 se activa por asociación directa con los ribosomas, asegurando que TORC2 permanece activo solo en células en crecimiento activo (Loewith and Hall, 2011). Por otro lado, existen indicios de que el estrés medioambiental, como el estrés por calor, reduce la actividad de TORC2, probablemente para prevenir el crecimiento en condiciones desfavorables (Jenkins et al., 1997; Kamada et al., 2005). Además ciertos estudios señalan que su actividad se ve regulada por sensores de estrés como la proteína calcineurina o la familia de proteínas Sml (Bultynk et al., 2006, Mulet et al., 2006) (Fig.1).

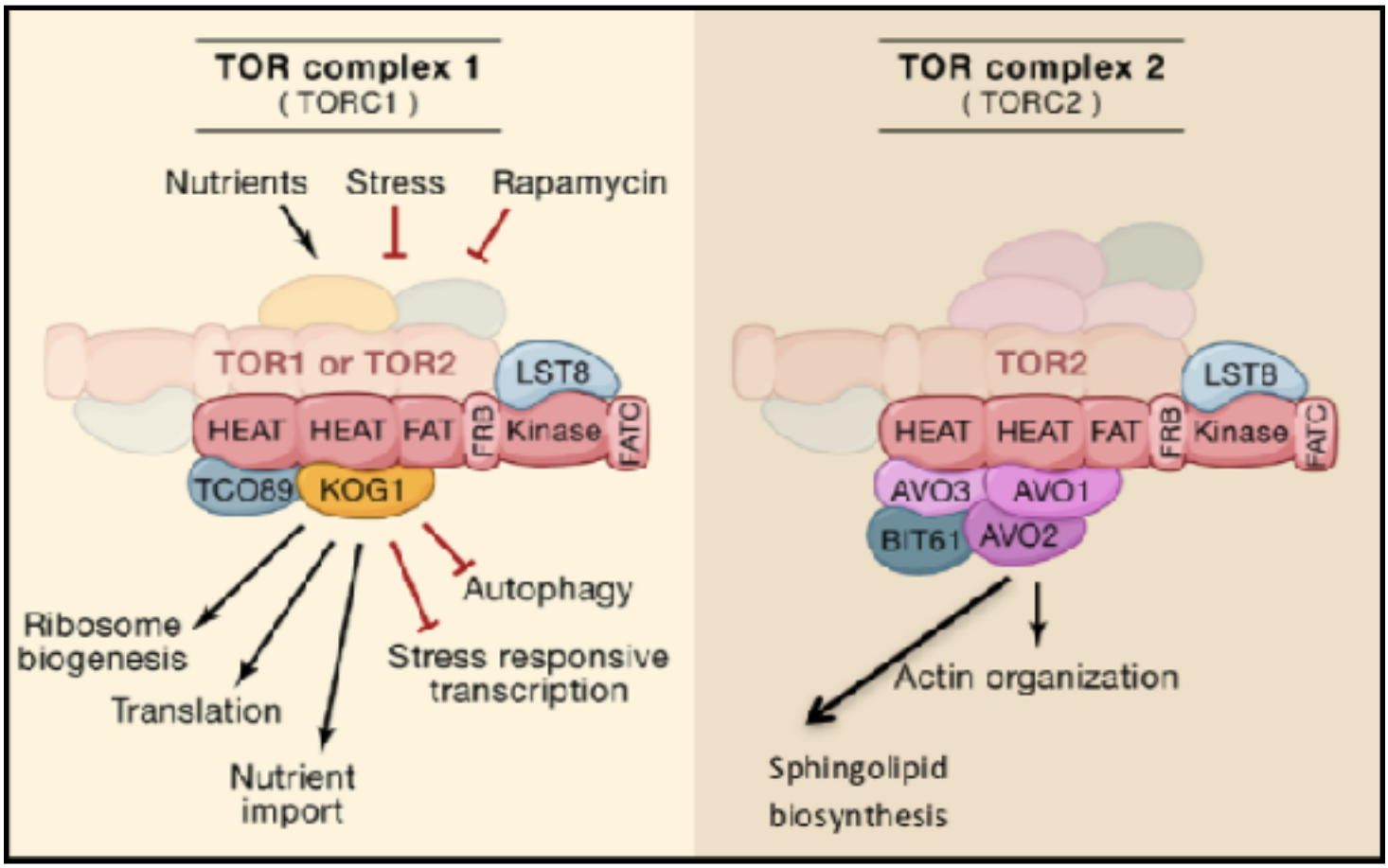

Figura 1. Representación de las proteínas asociadas a TOR (KOG1, TCO89, LST8, AVO1-3 y BIT61) y los dominios encontrados en TOR (HEAT, FAT, FRB, quinasa y FATC) en S.cerevisiae. *El complejo formado por TORC1 media la rama de señalización*

sensible a rapamicina que detecta las señales de crecimiento a la acumulación de masa. Los estímulos que regulan positivamente TORC1 y los procesos regulados por TORC1 que promueven la acumulación de masa se muestran con flechas negras. Las señales que regulan negativamente TORC1, como el estrés o la inanición y que inducen procesos regulados negativamente por TORC1 se indican en rojo. La rama de señalización de TORC2 es insensible a rapamicina y es imprescindible para la organización del citoesqueleto de actina y la síntesis de esfingolípidos (Adaptado de Wullschleger et al., 2006).

Al contrario que en *S. serevisiae,* en *Arabidopsis* sólo existe una copia de *TOR*, con un alto grado de similitud de secuencia y dominios estructurales con su homólogo en levadura. En plantas, *TOR* se expresa principalmente en los embriones y en el endospermo, aunque se encuentran ciertos niveles de expresión en todos los tejidos de la planta. Por otro lado, no se ha encontrado un homólogo de TORC2 en plantas, ya que componentes esenciales de dicho complejo son ausentes en sus genomas. TORC1 se ha asociado a funciones similares a las de levadura, como el control del ciclo celular, la síntesis de proteínas y el metabolismo en general, además del crecimiento. También regula negativamente procesos de reciclaje de nutrientes como la autofagia (Xiong et al., 2014).

En plantas, los estímulos que producen la activación de TOR son básicamente tres: el estado de la energética celular a través de la ruta SnRK1 (Baena-González et al., 2008), la acción de factores de crecimiento que activan la ruta PI3K (John et al., 2011) o la presencia de nutrientes que induce una serie de mecanismos de percepción y señalización intracelular (John et al., 2010). Estos factores se traducen en la modificación del complejo de inhibición TSC, lo cual activa a TOR. Una vez activado, TOR interacciona con diversas dianas aguas abajo, entre las cuales, las mejor caracterizadas e identificadas son S6K1 (*S6 Kinase 1*) y 4E-BP1 (*eukaryotic initiation factor 4E binding protein 1*) (Deprost et al., 2007; John et al., 2011). Ambas son proteínas reguladoras cuya fosforilación por parte de TOR produce un aumento de la síntesis de proteínas (Deprost et al., 2007).

TOR modula la síntesis de proteínas de muchos modos distintos en plantas, entre los que se encuentra la biosíntesis de ribosomas o del proceso de traducción (Xiong et al., 2014). Una de las vías de regulación se produce a través de su dominio quinasa, mediante la fosforilación de S6K1, de modo que activa genes ribosomales de síntesis de rRNA. Además, esta ruta promueve la iniciación y reiniciación de la traducción, así como

la agregación de los polisomas (Xiong et al., 2014). En levadura, existe un mecanismo adicional a través de TOR que regula el control de la traducción. GCN2, la quinasa objetivo de esta tesis doctoral, es responsable de este mecanismo, además del control general de aminoácidos (GAAC). A grandes rasgos, TOR fosforila la serina 277 de GCN2, impidiendo que este fosforile eIF2α a su vez, en respuesta a un déficit de aminoácidos en el medio intracelular. Aunque tanto TOR como GCN2 tienen homólogos en el genoma de *Arabidopsis*, se ha demostrado que ni en mutantes de sobreexpresión ni de silenciamiento de TOR se produce una diferencia significativa en la fosforilación de eIF2α entre ambas líneas cuando son expuestas a un herbicida que bloquea la síntesis de aminoácidos en plantas, indicando que TOR no está implicado en la respuesta al ayuno de aminoácidos (Lageix et al., 2008). Sin embargo, esto no significa que TOR y GCN2 puedan interaccionar en otras situaciones.

1.1.3.2 SnRK1

A los homólogos de SNF1 en plantas se les denomina SnRK1 (SNF-Related Kinase). Sus funciones son, en términos generales, muy similares a sus homólogos en levadura (SNF1) y en mamíferos (AMPk), siendo ambas proteínas unas quinasas altamente conservadas (Hey et al., 2010), aunque en plantas esta ruta es más compleja (Halford et al., 2004). Existe más de un gen con esta función en plantas, variando el número entre diferentes especies; en *Arabidopsis* la familia SnRK1 está compuesta por distintos genes. KIN10 y KIN11, son miembros de la familia SnRK1 dentro del genoma de *Arabidopsis* con el mayor grado de homología con SNF1 y AMPk (Baena-González et al., 2008). Están implicados en las rutas que regulan la protección y supervivencia de las plantas frente al estrés, la ausencia de luz y el ayuno de carbohidratos (Baena-González et al., 2007). Su función es coordinar la respuesta general frente a estos estímulos mediante cambios transcripcionales que abarcan todo el genoma, provocando la ralentización general de los procesos anabólicos junto con la promoción de los procesos catabólicos.

La ruta de señalización de las quinasas KIN10/KIN11 está mediada parcialmente por factores de transcripción tipo bZIP específicos como los DIN (*Dark-induced genes*) o los AREBP (*ABA response element binding protein*) (Hey et al., 2010). Los genes activados por estas quinasas pertenecientes a la familia SnRK1, codifican para proteínas implicados en diversas rutas catabólicas, como la degradación de la pared celular, la celulosa, la sacarosa, los lípidos o los aminoácidos, de forma que proporcionan una fuente de energía alternativa en los momentos en que la planta se encuentra expuesta a

situaciones de estrés. Además, inducen la activación de los genes *ATG*, los cuales promueven la autofagia (Baena-González et al., 2007). KIN10/11 se ven inhibidas por la glucosa-6-fosfato cuando los niveles de azúcares son altos, impidiendo la acción quinasa de SnRK1, no solo en enzimas relacionadas con el metabolismo de la glucosa sino con una amplia selección de genes a lo largo del genoma (Toroser et al., 2000). SnRK1 también responde a variaciones en los niveles de sacarosa, y activa la expresión de la sacarosa sintasa (Halford et al., 2004).

Aunque la ruta de traducción de señales no se conoce completamente, los estudios previos indican que, al igual que AMPk inactiva a TOR en mamíferos, KIN10/KIN11 es un inhibidor de TOR en plantas e induce la autofagia a través de la inactivación de dicha quinasa en este organismo (Baena-González et al., 2008, Xiong et al., 2014). En situaciones de estrés nutricional, falta de luz, u otro tipo de estreses (bióticos y abióticos), las SnKR1 inactivan TOR, el cual frena la síntesis de ribosomas y el inicio de la traducción. Es posible, como en el caso de TOR, que GCN2 y SnRK1 estén conectadas en una red de sensores de estrés intracelular (Hey et al., 2010). La siguiente figura recoge una visión conjunta de las vías de respuesta de las células vegetales a los diferentes tipos de estrés (Baena-González., 2009) (Fig. 2).

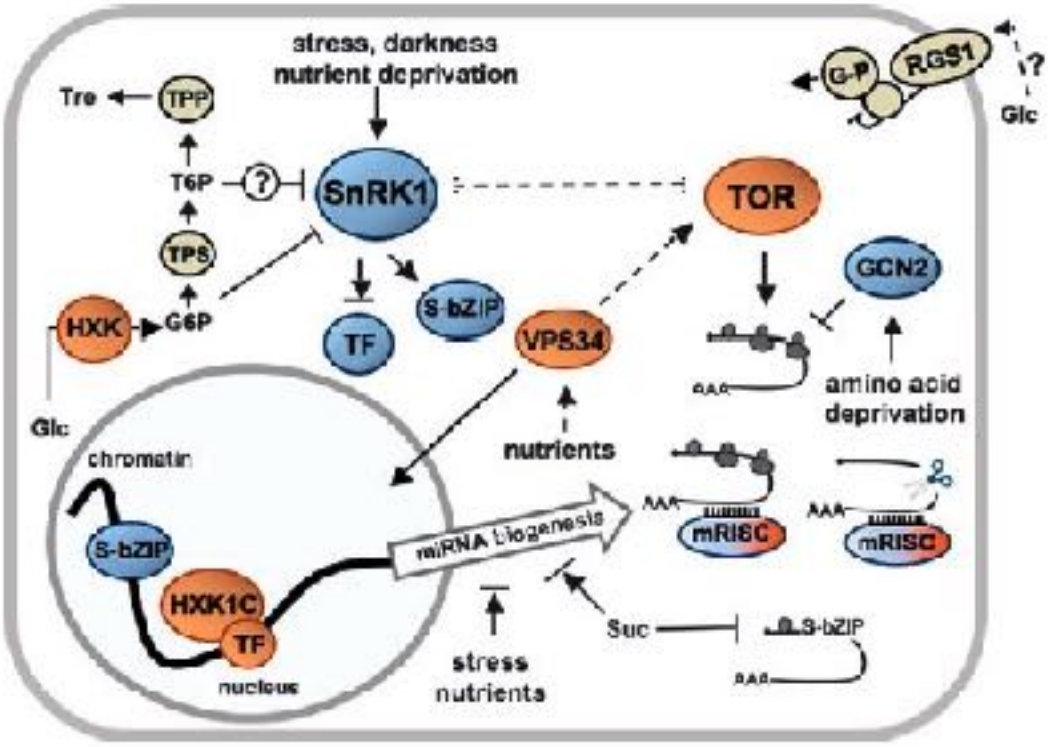

Figura 2. Conjunto de sistemas implicados en la percepción del estado energético y de la disponibilidad de nutrientes en las células vegetales*. Estos sensores pueden*

tener dos funciones antagónicas: una es inhibir la expresión de genes implicados en el anabolismo y el crecimiento celular (azul) y la otra es promover genes de reorganización de nutrientes en el caso de estados de estrés por inanición, como SnRK1, que ponen en marcha factores de transcripción (TF y bZIP), que expresan una gran cantidad de genes; o como GCN2, que en situaciones de ayuno de aminoácidos impide el inicio de la traducción fosforilando a eIF2a. Los azúcares tales como la glucosa (G6P), la trehalosa (T6P) y la sacarosa (Suc) reprimen la actividad inhibitoria de SnRK1 en el caso de los primeros, o actúan inhibiendo la expresión de miRNA por parte de los bZIP, en el caso de la glucosa. La segunda consiste en utilizar la disponibilidad de nutrientes para impulsar el crecimiento, como TOR, que cuando está activada promueve la síntesis de ribosomas y la traducción (Adaptado de Baena-González., 2009).

1.1.4. Control traduccional en respuesta al estrés

La traducción es el proceso bioquímico más complejo e intenso que soportan las células en crecimiento. Requiere de un gran aporte energético y de un suministro estable de los veinte aminoácidos que las conforman. Además, estas proteínas recién formadas deben plegarse, ensamblarse, modificarse covalentemente y posicionarse en su lugar correcto dentro de la célula (Roy y Arnim et al., 2013). Por todo ello, no es de extrañar que una de las principales respuestas de las células frente a condiciones de estrés sea el cese parcial o prácticamente total de los procesos que impliquen un consumo energético y que además son vitales para la homeostasis, incluida la transcripción y la síntesis de proteínas. La traducción es uno de los principales procesos que deben inhibirse en respuesta a la mayoría de estreses celulares. Sin embargo, bajo condiciones donde la síntesis global de proteínas se encuentra prácticamente inhibida, algunas proteínas son capaces de sintetizarse como parte de los mecanismos de supervivencia celular, estas proteínas pueden mitigar el daño causado por el estrés y permitir que las células toleren las condiciones estresantes con mayor eficiencia (Holcik y Sonenberg et al., 2005).

En comparación con la regulación transcripcional, el control de la traducción de los mRNA existentes, permite cambios más rápidos en las concentraciones celulares de las proteínas codificadas por la utilización diferencial de los mRNA ya preexistentes. Este hecho puede garantizar una rápida y eficiente producción de productos genéticos sin tener que esperar a la síntesis y procesamiento del RNA. Por tanto se pueden utilizar para

mantener la homeostasis celular, además de modular cambios más permanentes en la fisiología de la célula (Rhoads et al., 1993; Sonenberg y Hinnebusch et al., 2009).

La decodificación del mRNA por el ribosoma es un proceso complejo que requiere un gran número de factores de iniciación, elongación y terminación, algunos de los cuales sirven como puntos finales para la regulación de la traducción. El control traduccional de la expresión génica se produce principalmente a nivel de iniciación (Roy y Arnim et al., 2013). Dos eventos clave del control de la traducción son la asociación entre el mRNA con extremo el 5‘ y el complejo de preiniciación, y la unión del tRNA de iniciación con el codón de inicio. Ambos eventos están mediados por factores de iniciación eucariotas que están regulados por las quinasas efectoras e inhibidoras.

Como se muestra en la Figura 3, la traducción es un proceso complejo el cual incluye muchos elementos, que de forma conjunta organizan la expresión génica celular. Uno de los pasos principales en los que se regula la traducción durante el estrés es la fosforilación de la subunidad alfa del factor de iniciación eIF2 (Deng et al., 2002). Tras la fosforilación por las quinasas sensibles al estrés, eIF2α afecta la tasa global de iniciación de la traducción. El mecanismo propuesto se explicará con más detalle en la siguiente sección, y se centrará en el papel que desempeña la proteína quinasa GCN2 en la fosforilación de eIF2α (área marcada en verde) y en qué condiciones se produce esta fosforilación.

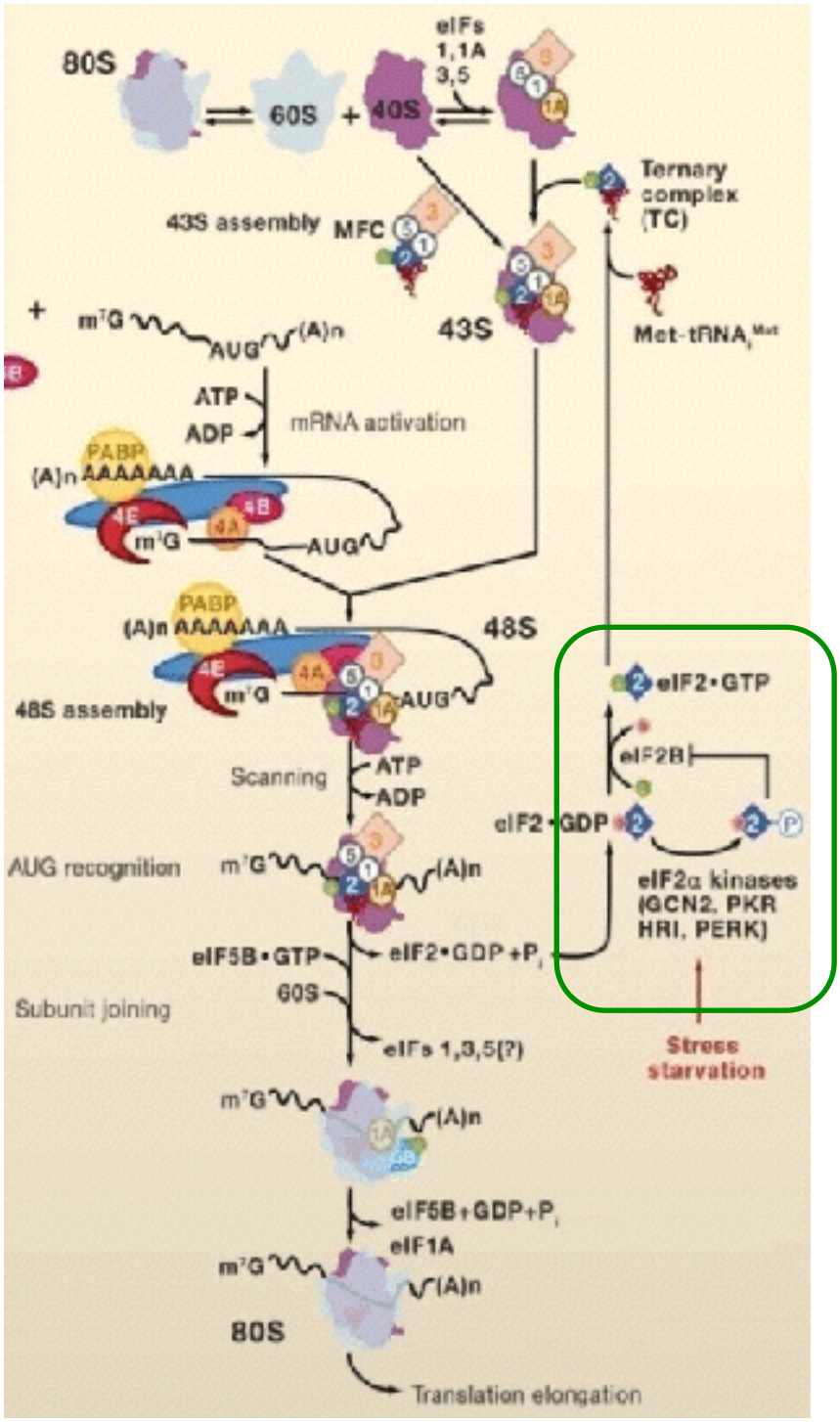

Figura 3. Esquema general del proceso de traducción. *En la zona marcada en verde se ve reflejado el papel de eIF2a en el proceso (Adaptado de Sonenberg y Hinnebusch, 2009).*

1.1.5. Papel de eIF2 en la traducción

Antes de que comience el inicio de la traducción, eIF2 debe unirse a GTP y al iniciador Met-tRNAiMet como un complejo ternario (TC). El TC se unirá a la subunidad ribosomal

40S y comprobará el mRNA hasta que reconozca el anti-codón Met-tRNAiMet y los pares de bases AUG con el codón de inicio. Tras el emparejamiento de bases con AUG, eIF2-GTP se hidroliza a eIF2-GDP y se libera del TC. Para reconstituir el TC para otra ronda de iniciación de la traducción, eIF2-GDP debe ser reciclado nuevamente a la forma activa eIF2-GTP por el factor de intercambio de guanina eIF2B. Sin embargo, la fosforilación de eIF2α inducida por el estrés bloquea la reacción de intercambio de GTP, ya que el eIF2-GDP fosforilado es un inhibidor competitivo de eIF2B. Como eIF2 está presente en niveles más altos que eIF2B, solo se necesita un pequeño número de moléculas eIF2α fosforiladas para unirse e inhabilitar las moléculas eIF2B, lo que evita el intercambio de GDP a GTP (Dever et al. 1995). El resultado final es menos eIF2-GTP disponible para formar el TC y comenzar otra ronda de inicio de la traducción, además de regular a la baja la síntesis de proteínas (Fig.4) (Immanuel et al 2012).

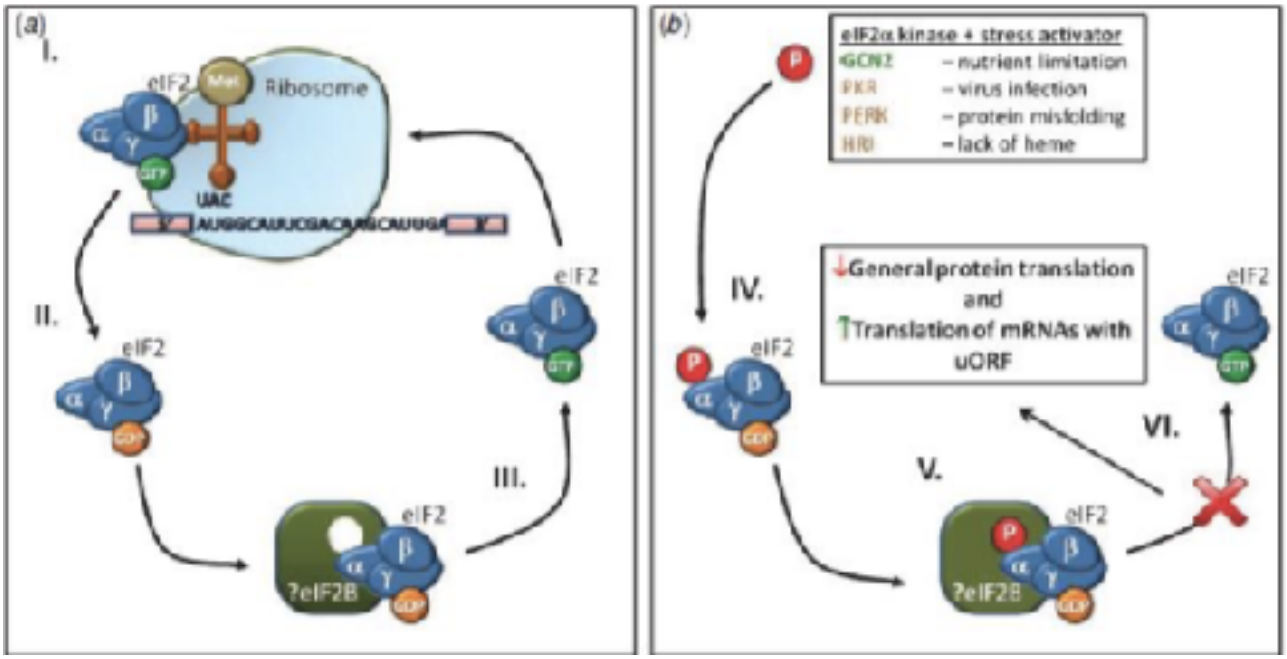

Figura 4. Ruta de regulación de la fosforilación de eIF2α. *(a) El inicio de la traducción comienza con la unión de GTP a eIF2 y el iniciador Met-tRNAMet formando un complejo ternario. (b) Cuando ciertos estreses activan una quinasa eIF2a, eIF2a es fosforilado (Immanuel al 2012).*

La fosforilación de eIF2α en vertebrados se lleva a cabo por una familia de cuatro quinasas (Fig. 5), PKR (protein kinase R/RNA-dependent proteína kinase) o proteína quinasa regulada por el RNA de doble cadena, PERK (PKR-like endoplasmatic reticulum) o pro-

teína quinasa regulada por estrés en el retículo endoplásmatico, GCN2 (general control non-derepressible-2) o proteína quinasa regulada por limitación de nutrientes, y HRI (heme regulated inhibitor) o inhibidor regulado por hemina (Donnelly et al., 2013). Las cuatro quinasas eIF2α comparten un dominio de proteína quinasa altamente conservado, pero difieren en sus dominios reguladores, lo que permite que cada quinasa responda a estímulos diferentes (Byrne et al., 2012). En mamíferos, GCN2 es la quinasa eIF2α implicada en respuesta a la limitación de nutrientes (Zhang et al 2002), PERK modula la expresión génica en respuesta al plegamiento incorrecto de las proteínas en el retículo endoplásmico (ER) (Harding et al., 2002), PKR participa en la vía antiviral mediada por el interferón (Clemens et al., 1997) y el HRI coordina la síntesis de proteínas a la disponibilidad del grupo hemo en el linaje de células eritroides (Chen et al., 2007). Por el contrario, GCN2 es la única quinasa eIF2α en plantas (Lageix et al., 2008) y también es la única presente en *S. cerevisiae* (Wek et al., 2006; Muñoz et al., 2011) (Fig. 5). Todas estas quinasas fosforilan específicamente a eIF2α en un residuo de serina conservado (Ser51 en conejo y Drosophila; Ser52 en levadura, ratón y humanos; Ser56 en *Arabidopsis*) (Hinnebusch et al., 2005) para inhibir la traducción de proteínas.

Figura 5. Esquema de las quinasas eIF2a distribuidas entre los organismos eucariotas. *(Adaptado de Segovia de Arana et al., 2007)*

Como se mencionó anteriormente, la síntesis de proteínas se regula parando la traducción cuando se produce la fosforilación de eIF2α, pero permitiendo por otro lado la traducción de elementos como el factor de transcripción 4 (ATF4) en mamíferos y GCN4 en levaduras. Tomando ATF4 como ejemplo, este factor de transcripción regula al alza numerosos genes involucrados en la homeostasis de los aminoácidos, el metabolismo redox

y la apoptosis. La expresión de ATF4 en condiciones de detención global de proteínas implica dos marcos de lectura abiertos en sentido ascendente (uORF) (Fig. 6) ubicados en el extremo 5 'del mRNA de ATF4 que facilitará la traducción de este activador transcripcional cuando eIF2 esté fosforilado (Wek et al., 2006). Los dos uORF contribuyen de manera diferencial a la expresión de ATF4 (Vattem et al., 2004). El uORF1 es un elemento que facilita la exploración y reiniciación del ribosoma en las regiones de codificación aguas abajo del mRNA de ATF4. Cuando eIF2-GTP es abundante en células no estresadas, los ribosomas que exploran la región aguas abajo del uORF1 se reinician en la siguiente región de codificación: uORF2, es un elemento inhibidor que bloquea la expresión de ATF4. En condiciones de estrés, la fosforilación de eIF2 y la reducción resultante en los niveles de eIF2-GTP, aumentan el tiempo requerido para que los ribosomas sean competentes para reiniciar la traducción. Esta reiniciación retardada permite que los ribosomas reconozcan el inhibidor uORF2 y, en su lugar, se reinicie en la región de codificación ATF4. Un mecanismo similar ocurre para la traducción de GCN4 en levadura (Hinnebusch, 2005).

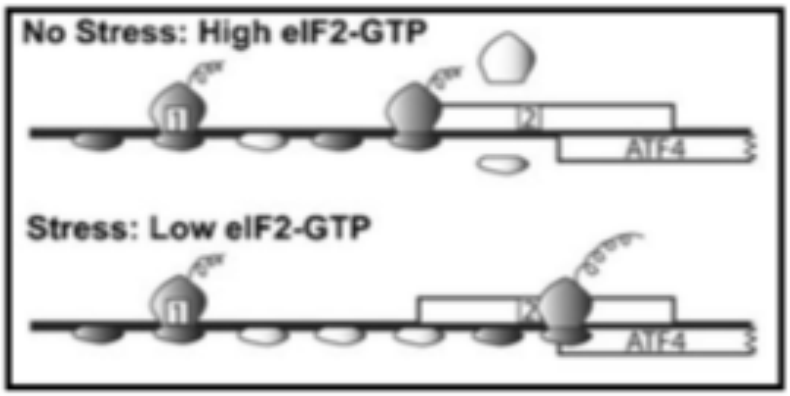

Figura 6. La fosforilación de eIF2*. Dicha fosforilación reduce los niveles de eIF2-GTP y facilita la derivación del uORF2 inhibitorio, lo que mejora la traducción de la región codificadora de ATF4 en respuesta a las condiciones de estrés (Wek et al., 2006).*

1.2. Ruta GCN2: Respuesta al estrés por ayuno de aminoácidos

1.2.1. GCN2. *General Control Non-derepressible-2*

GCN2 se descubrió inicialmente como un sensor de la disponibilidad de aminoácidos y un regulador de los cambios en la expresión génica en respuesta al ayuno de aminoácidos.

La escasez de aminoácidos produce en la célula la acumulación de tRNAs no cargados (tRNAdeacyl). Estos son detectados por GCN2, lo que lleva a la estimulación del dominio catalítico de la proteína quinasa y por consiguiente a la fosforilación de eIF2α (Dever et al., 1993).

En *S.cerevisie*, GCN2 está formada por 1659 aminoácidos (Hinnebusch, 2005). Tiene 4 dominios principales en su estructura, del N-terminal al C-terminal: un dominio N-terminal llamado RWD, un dominio pseudoquinasa sin función enzimática conocida, el dominio catalítico de quinasa eIF2α, un dominio con similitud a histidil-tRNA sintetasa que se une a tRNA descargado y un dominio de dimerización C-terminal (CTD) y de unión a ribosoma (Hinnerbusch et al., 2005) (Fig.7). Otras características de su estructura es su dominio N-terminal altamente conservado y una región cargada (con gran densidad de aminoácidos cargados positiva y negativamente), ambas sin función determinada (García-Barrio et al., 2000)

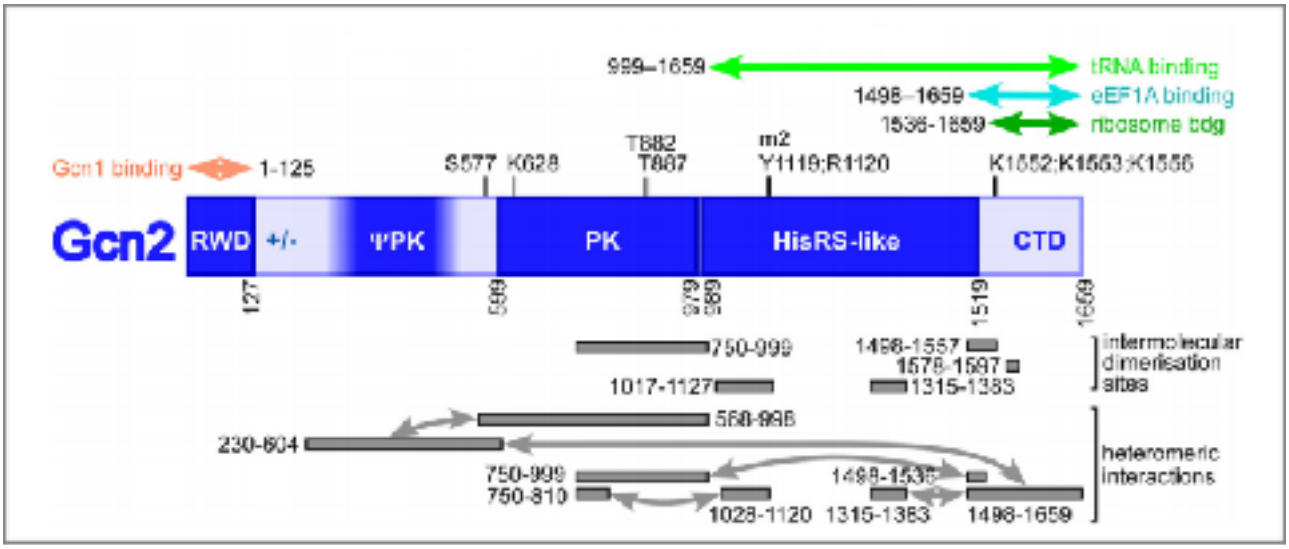

Figura 7. Representación esquemática de los dominios de GCN2 en levadura. *Se muestran los dominios estructurales de la quinasa, y los aminoácidos con función conocida. A continuación, se representan las regiones que realizan las dimerizaciones intermoleculares, es decir, se unen a una misma región de una segunda molécula de GCN2, y las regiones de interacciones heteroméricas. En color, aparecen las regiones de unión a GCN1, tRNA, el regulador negativo eEF1A y a los ribosomas (Adaptado de Castilho et al., 2014).*

La activación de la quinasa se logra mediante la reorganización de las interacciones entre los residuos de aminoácidos específicos cuando un tRNA descargado se une al dominio HisRS / C-terminal (Castilho et al., 2014).

En condiciones de estrés provocado por la limitación de aminoácidos, o en condiciones de ayuno, GCN2 y su interactor GCN1 forman un complejo trimérico con el ribosoma. A este complejo se le asocia, a través de GCN1, una tercera proteína llamada GCN20, la cual también forma parte de este complejo.

Cuando los tRNA descargados se acumulan en la célula, el sitio-A de la traducción de los ribosomas puede acomodar a un tRNA descargado afín. Este tRNA descargado luego se transfiere desde el sitio-A a GCN2. El tRNA descargado se une al sitio-A antes de ser detectado por GCN2, de forma que GCN2 detecta la señal de inanición, en lugar de los tRNA descargados como ocurre en condiciones normales durante el proceso de traducción. La posible función de GCN1 puede ser la de entregar o facilitar la unión de los tRNA descargados al sitio-A, o la de transferir los tRNA descargados del sitio-A a GCN2. Alternativamente, es posible que GCN1 sea una proteína de soporte que posicione a GCN2 en el ribosoma de tal manera que los tRNA descargados puedan transferirse directamente desde el sitio-A a GCN2 (Castilo et al., 2014) (Fig. 8).

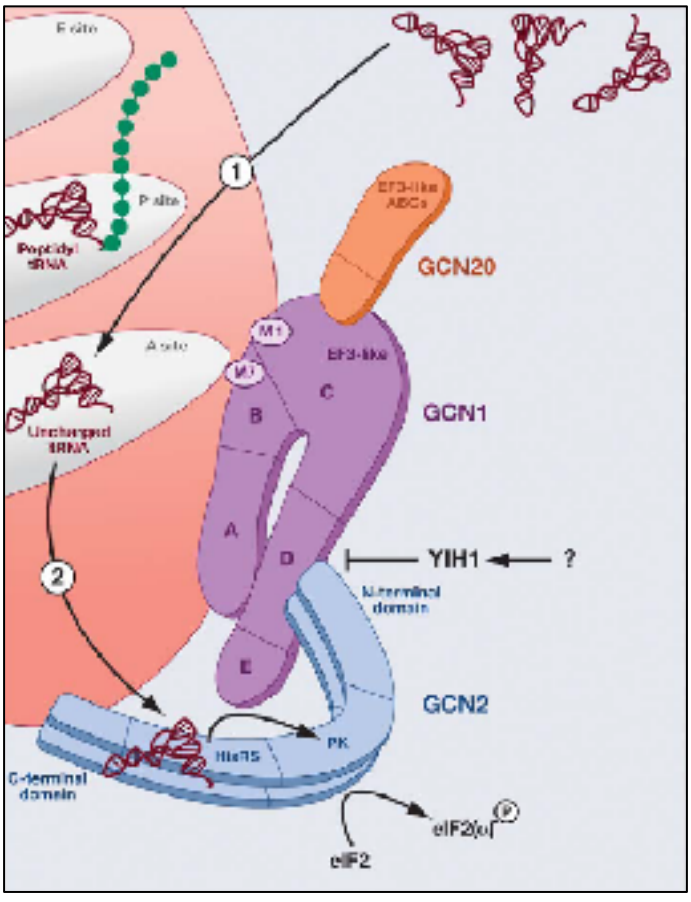

Figura 8. Esquema del modelo de interacción del complejo GCN1/GCN20 con GCN2. *El sustrato de GCN2 son los tRNAs no cargados que entran al sitio A (1), que al unirse al dominio HisRS (2) éste cambia la conformación de la región quinasa, que a su vez fosforila a eIF2a. GCN1/GCN20 funcionan como EF3 en E.coli facilitando la unión de los tRNA al sitio A, y estarían físicamente asociados entre sí, al ribosoma y a GCN2. YIH1 actúa inhibiendo esta última unión, aunque la señal fisiológica que lo induce aún es desconocida (Adaptado de Hinnebusch, 2005).*

GCN2 también se activa por otros estreses diferentes a la inanición de aminoácidos. En levadura, se demostró que GCN2 se activa en respuesta a la privación de purinas (Rolfes and Hinnebusch et al., 1993), la limitación de la glucosa (Yang et al.,2000), aumento de la salinidad (Goossens et al., 2001), exposición al metil metanosulfonato (MMS) (Hinnebusch et al.,2002), al antibiótico rapamicina, el cual inhibe la ruta de TOR (Cherkasova et al., 2003), la infección viral o la radiación UV (Deng et al., 2002; Berlanga et al., 2006). Otros estudios han demostrado también la participación de la señalización de GCN2 en la progresión del cáncer y en las respuestas celulares a los agentes terapéuticos clásicos. Se ha demostrado que GCN2 puede activarse por hipoxia (Liu et al., 2010), al menos en células murinas in vitro; de manera más significativa, la activación de GCN2 ha demostrado ser una consecuencia no solo de la privación de aminoácidos sino también de la privación de glucosa, dos estreses comúnmente encontrados por los tumores sólidos, en células cancerosas humanas (Ye et al., 2010), aunque el mecanismo exacto aún no se ha definido.

1.2.2. Mecanismos de control de GCN2

Como se ha comentado anteriormente, los complejos TOR (TORC1 y TORC2) realizan una función principal en el control de rutas implicadas en la defensa de células sometidas a estrés por ayuno. Existe evidencia de que cuando no está activada, GCN2 presenta un residuo fosforilado, Ser-577, entre los dominios pseudoquinasa y quinasa. En presencia de rapamicina, los niveles de Ser-577P disminuyen, por lo que parece indicar que la función activadora de eIF2α por parte de TOR se realiza promoviendo la fosforilación constitutiva de GCN2. La Ser-577P es defosforilada por una fosfatasa desconocida, aunque se tiene la impresión de que esta función la realiza una fosfatasa de eIF2α, Sit4p. A través de la proteína Tap4p, TOR inhibiría la función defosforiladora de Sit4p, modelo apoyado por el hecho de que la rapamicina disocia el complejo Tap4p/Sit4p in vivo (Cherkasova et al., 2003). En presencia de estímulos de estrés, TOR se inactivaría,

aumentando la afinidad por los tRNA no cargados al inducir un cambio conformacional por la defosforilación de la Ser-577, e iniciando la respuesta del control general a través de GCN2 (Hinnebusch, 2005).

Snf1, el homólogo de SnRK1 en plantas o AMPK en mamíferos, realiza una función central de sensor del estado energético de la célula, y también se ve activada por el ayuno de aminoácidos. Por una vía que aún se desconoce, Snf1 impulsa la autofosforilación de GCN2 y la fosforilación de eIF2α por parte de éste en experimentos de ayuno con histidina. Aunque no se ha demostrado que Snf1 fosforile a GCN2 en vivo, ambas proteínas co-precipitan en ensayos de inmunoprecipitación, indicando que la regulación de Snf1 es necesaria para la correcta activación de GCN2 (Castilho et al., 2014).

Otros mecanismos de regulación involucran factores de elongación de la traducción. Eef3 regula la salida de los tRNA del sitio E mediante el uso de ATP a través de sus dominios ABC, tras lo cual eIF1A transporta otro tRNA al sitio A. En condiciones de abundancia de aminoácidos, se produce la síntesis de proteínas, y la presencia de eEF3 unido al ribosoma impide que GCN1 (que tiene un dominio similar a EF3) se una, inhibiendo la función de GCN2. Además, eIF1A se une al dominio CTD de GCN2 e impide la fosforilación de eIF2α (Castilho et al., 2014).

1.3. GCN2 en plantas

El homólogo de GCN2 en *Arabidopsis*, AtGCN2, presenta un dominio quinasa de eIF2α junto a un dominio HisRS, aunque es más pequeño, su secuencia contiene 1241 aminoácidos (140 kDa), y fosforila a eIF2α en la serina 52 en respuesta a diversos estreses abióticos. AtGCN2 complementa al mutante *gcn2* de levadura, corroborando la existencia de una versión del GAAC en plantas (Zhang et al., 2003; Lageix et al., 2008). EST (Expression sequence Tags) similares a GCN2 han sido encontradas desde entonces en diferentes especies vegetales tales como arroz, trigo, cebada, patata, soja, remolacha azucarera, caña de azúcar, alfalfa, algodón, álamo, cebolla, loto, y Zinnia (Halford, 2006).

Aunque experimentos con ayuno de aminoácidos demuestran la existencia de un mecanismo de control general y la implicación de AtGCN2, existen numerosas diferencias entre *S. Cerevisiae* y *Arabidopsis*. La más destacable es que no se ha

encontrado un homólogo de GCN4 en plantas (Hinnebusch, 2005). Esto indica que existen otros mecanismos, independientes de la fosforilación de eIF2α, que están implicados en la respuesta al ayuno de aminoácidos, y que GCN2 podría estar implicado en otras funciones dentro de la planta (Halford et al., 2006).

Se han localizado homólogos de GCN1 y GCN20 en *Arabidopsis* los cuales serán descritos posteriormente.

1.3.1. Función de GCN2 en plantas. Control de la síntesis de proteínas

AtGCN2 cumple con el rol de organizador en el control de la traducción en respuesta a estreses, disminuyendo la síntesis de proteínas mediante la fosforilación de eIF2α, ostentando un rol similar a su homólogo en levadura. Este papel ha sido estudiado principalmente en experimentos de estrés por ayuno de aminoácidos inducido por inhibidores de las rutas biosintéticas, mayormente herbicidas, como el glifosato.

El glifosato (N-(fosfometil) glicina), o roundup, es un herbicida desarrollado por Monsanto que afecta a la "5-enolpyruvylshikimate-3-phosphate-synthase" (EPSPs; EC 2.5.1.19), impidiendo el desarrollo de la ruta del ácido siquímico, precursor de todos los aminoácidos aromáticos, quinonas y varios cofactores. Otros autores señalan la acumulación del ácido siquímico como la causa de la toxicidad producida por este herbicida (Fischer et al., 1986; De María et al., 2006). La exposición a glifosato debilita lentamente a la planta, produciéndose la muerte de la misma en días o semanas en función de la dosis (Duke et al., 2008).

La disminución de los niveles de aminoácidos aromáticos por el glifosato induce la fosforilación por AtGCN2 de eIF2α (Zhang et al., 2008b), observada también en respuesta a otros estreses tales como: la invasión de virus (Zhang et al., 2008), exposición a UV-C, shock por frío, daño a la planta, metil jasmonato, ácido 1-aminociclopropánico-1-carboxílico, ácido acetilsalicílico y 8-azaadenina (Lageix et al., 2008). El ácido jasmónico (JA) y los compuestos de señalización relacionados, como el metil jasmonato, son señales de posibles lesiones tisulares y de una posterior activación de la respuesta en defensa a los insectos (Lageix et al. 2008). Algunos de estos estreses podrían conducir a la acumulación de tRNAs no cargados.

Existen aún muchas preguntas sin responder alrededor del efecto del glifosato y el papel jugado por GCN2. Aún no se conocen bien los mecanismos a través de los cuales GCN2

produce la respuesta al estrés inducido por el herbicida, ni qué procesos son modulados en esta respuesta. Al contrario que pasa en organismos tales como *E.coli* (Lu et al., 2013), *Soja* (Zhu et al., 2008) o *Festuca* (Cebeci et al., 2009), donde el tratamiento por glifosato provoca la respuesta de gran cantidad de genes, análisis de hojas en *Arabidopsis* solo mostraron 16 genes cuya respuesta fue modulada por el herbicida (Malay et al., 2009). Esta diferencia, junto con el hecho de que AtGCN2 está implicado sólo de forma parcial en la respuesta al ayuno de aminoácidos (Zhang et al., 2008), y que recientemente se ha demostrado que la fosforilación de eIF2α no está correlacionada con la inhibición global de la síntesis de proteínas (Izquierdo et al., 2018) parece indicar la existencia de otros mecanismos involucrados en la defensa contra el herbicida. Todo esto, junto con la ausencia de un homólogo de GCN4 en plantas, sugiere que la regulación de la traducción a través de eIF2α puede ser una vía secundaria en plantas.

1.3.2. GCN2 y la luz ultravioleta

Se ha demostrado que la irradiación con luz UV-C produce la fosforilación de eIF2α a través de la activación de GCN2 en mamíferos (Deng et al. 2002), lo que resulta en una detención de la traducción (Grallert y Boye, 2007). Además, Zhong y sus colaboradores (2011) vieron que la luz UV-A, UV-B y UV-C inducen la fosforilación de eIF2α en función de la dosis y el tiempo de exposición a través de distintos mecanismos de señalización regidos por diferentes familias quinasas, incluyendo GCN2.

Varios estudios han demostrado que GCN2 afecta a la progresión del ciclo celular al retrasar la formación del complejo de pre-replicación, un paso obligatorio en la preparación para la replicación del DNA (Tvegard et al., 2007; Krohn et al., 2008) después de la exposición a la radiación UV-C. Aún se desconoce cómo GCN2 puede ser activado por la luz UV o cuáles son sus sustratos que median la regulación del ciclo celular (Grallert y Boye, 2007). En principio, la fosforilación de eIF2α por irradiación de luz UV-C solo tienen relación a través de GCN2, ya que no se han encontrado otras evidencias de este suceso en células de mamífero irradiadas por UV (Deng et al., 2002). Algunos autores sugieren que la luz UV media la unión de los tRNA y GCN2, para así activar a GCN2. Otros estudios sugieren que la activación de GCN2 por luz UV en células de mamíferos puede deberse al rápido consumo de arginina para producir óxido nítrico (Lu et al., 2009). En mamíferos se ha observado que el sustrato de GCN2 cuando es estimulado por radiación

UV es la metionil-tRNA sintetasa (MRS) (Lee y Kim, 2011). GCN2 parece fosforilar el dominio de unión entre el tRNA y el sustrato MRS. Esos estudios sugieren que la fosforilación reduce la unión entre el tRNA-Met y el sustrato MRS, y la radiación UV disminuye los niveles de Met-tRNA cargado in vivo (Castilho et al., 2014).

En 2008, Lageix et al demostraron que GCN2 fosforila eIF2α en *Arabidopsis* en respuesta a la irradiación con UV-C (Fig. 9A). eIF2α se fosforila rápidamente después de la irradiación con UV-C. Esta respuesta comienza solo 20 minutos después de la irradiación, con un máximo de fosforilación después de 1 hora y un retorno a los niveles basales después de 6 horas (Fig. 9B). Ambos eventos de fosforilación dependen completamente de la presencia de GCN2, ya que no se pudo detectar la fosforilación de eIF2α en la línea de GCN2 mutante en condición de estrés (Fig. 9A).

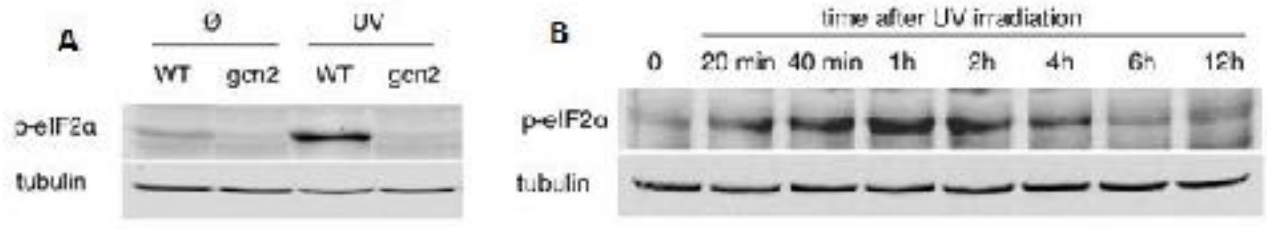

***Figura 9*. Fosforilación de *eIF2a en Arabidopsis Thaliana*.** *A) Fosforilación de eIF2a en plántulas de tipo silvestre (WT) y en plántulas mutantes gcn2 no tratadas (Ø) o irradiadas con UV-C (UV). B) AtGCN2 fosforila a eIF2a después de ser irradiada con UV-C en Arabidopsis Thaliana (Lageix et al., 2008).*

1.4. GCN1 en plantas

GCN1 fue la primera proteína encontrada para promover la función de GCN2. En levadura se comprobó como una cepa mutante *gcn1* (cepa *gcn1Δ)* era incapaz de activar a GCN2 tras un estrés por ayuno de aminoácidos. Sin embargo, la actividad quinasa de GCN2 se podía detectar en dicha cepa mutante, lo que sugirió que GCN1 no era necesaria para la actividad de la quinasa per se, sino que lo era para la activación in vivo de GCN2 en respuesta a la falta de aminoácidos (Marton et al., 1993). Todos los datos obtenidos in vivo en levadura avalan la idea de que GCN1 es necesario para que GCN2 sea capaz de detectar al tRNA descargado.

GNC1 es una proteína formada por 2672 aminoácidos, entre los aminoácidos 1330-1641 presenta homología con la zona N-terminal del dominio de repetición HEAT del factor 3 de elongación de la traducción fúngica (eEF3) (Fig.10) (Marton et al., 1993). En levaduras, este dominio es requerido para la liberación del tRNA descargado desde el sitio E ribosómico durante la biosíntesis de proteínas. La misma región C-terminal es determinante para la interacción con GCN2 (Sattlegger e Hinnebusch, 2000). Otras proteínas que también contienen estos dominios propuestos para servir como sitios de interacción para otras proteínas son: la proteína huntingtina, el factor de alargamiento 3, la fosfatasa 2A y TOR1 (Sattlegger e Hinnebusch, 2000).

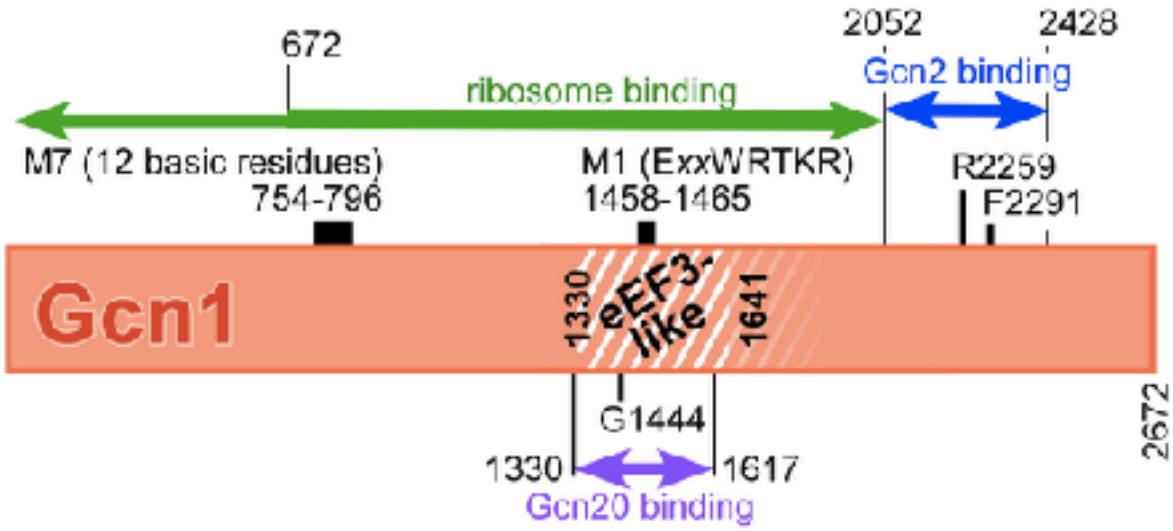

Figura 10. Representación esquemática de los dominios de GCN1 en levadura. *La parte media de GCN1 presenta homología con la región N-terminal de eEF3 (sombreado). Las zonas remarcadas con flechas dobles en morado (1330-1617) son necesarias para la unión de ribosomas, y es el lugar de unión con GCN20. Las zonas indicadas con flechas dobles en azul (2052-2428) son la zona de unión de GCN2 (Adaptado de Castilho et al., 2009).*

En *Arabidopsis*, la única proteína en el genoma que presenta similitud con GCN1 (57% de similitud sobre la región más conservada C-terminal) es la proteína ILITHYIA (ILA). Inicialmente fue identificada como una proteína necesaria para la embriogénesis (Johnston et al., 2007), pero también está relacionada con la inmunidad de las plantas frente a infecciones provocadas por bacterias. En concreto, se ha demostrado que la proteína ILA

es necesaria para controlar las posibles infecciones contra *Pseudomonas syringae*, así como la resistencia condicionada por genes de resistencia específica (R), inmunidad producida por efectores (ETI) y las resistencias sistemáticas adquiridas (SAR) (Monaghan y Li, 2010). ILA es una proteína larga formada por 2696 aminoácidos, su estructura también contiene repeticiones HEAT en su región media.

Los fenotipos que muestran los mutantes de *ila* incluyen hojas amarillas con formas aberrantes y esterilidad masculina, lo que indica un papel pleiotrópico en el desarrollo de las plantas (Monaghan y Li, 2010). Por otro lado, se ha visto la participación de ILA en la mediación de la fosforilación de eIF2α a través de la activación de GCN2 (Wang et al., 2016).

Para poder conocer el papel que desempeña la proteína ILA se obtuvieron mediante genética inversa tres alelos de inserción de T-DNA, Salk_149084 (*ila-2*) y Salk_119854 (*ila-1*) llevan inserciones en los exones 22 y 24, respectivamente, mientras que Salk_041123 (*ila-3*) lleva una inserción en el exón 54 de *ILA* (Fig. 11a). A nivel fenotípico los mutantes *ila-1* e *ila-2* presentan un tamaño más pequeño que su tipo silvestre Col-0 y además presentan hojas aserradas y de color amarillo a verde claro, especialmente en las hojas emergentes (Fig. 11b). Por el contrario, el alelo *ila-3* solo presenta las hojas ligeramente aserradas, y de color más verde en comparación con los otros alelos. Sobre la fertilidad de los mutantes *ila*, los alelos *ila-1* e *ila-2* son estériles y deben ser propagados en heterocigosis (Monaghan y Li, 2010).

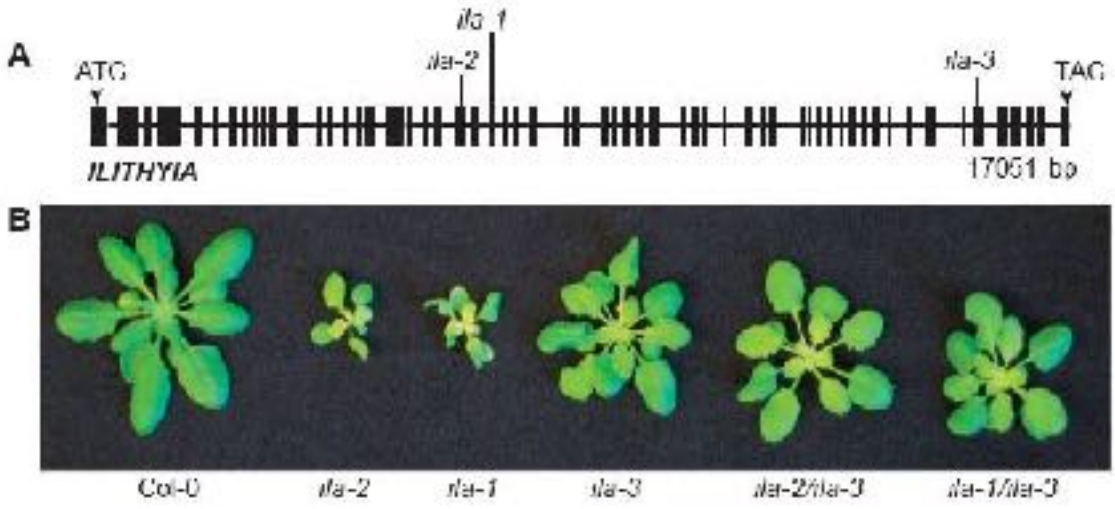

Figura 11. Estructura y morfología de GCN1 en Arabidopsis Thaliana. *A) Estructura génica de la inserción de T-DNA en los mutantes ILA(At1g64790). B) Morfología de la*

*planta silvestre Col 0 y los mutantes ila-1, ila-2 e ila-3 así como los cruces obtenidos usando ila-3 como macho en ambos casos. (*Monaghan y Li, 2010)

También se demostró que la coloración amarillenta de las hojas en los mutantes *ila-1* e *ila-2* es debido a unos niveles mucho más bajos de clorofila A y B en comparación con el tipo silvestre. Las plantas *ila-3* también presentaron niveles inferiores respecto al tipo silvestre, pero notablemente más altos que en los mutantes *ila-1* e *ila2*. Las deficiencias de clorofila pueden estar relacionadas con la disminución en la expresión del complejo LHCB1 (*light harvesting complex B1)* (Lopez-Juez et al., 1998). La expresión de este gen en los mutantes *ila-1* e *ila-2* fue inferior en comparación con Col-0, demostrando que el fenotipo de hojas amarillas presente en los mutantes ILA podría estar relacionado con la disminución de los niveles de clorofila y la expresión de LHCB1 (Monaghan y Li, 2010).

1.5. GCN20 en plantas

GCN20 actúa junto con GCN1 como un efector positivo facilitando la activación de la quinasa. El dominio N-terminal de GCN20 se une al dominio central eIF3 de GCN1, modulando así su actividad (Marton et al., 1997; Garcia-Barrio et al; 2000). GCN20 pertenece a una subfamilia de proteínas transmembrana que poseen dominios o casetes de unión a ATP y que utilizan la energía generada por la hidrólisis del ATP para mover sustratos entre ambos lados de la membrana. Además, GCN20 forma parte de la familia ABC y sus regiones de unión a nucleótidos, los cuales ocupan un 80% de su estructura a partir del C-terminal, son muy similares a EF3 (Vazquez de Aldana et al., 1995).

En *Arabidopsis* hay 26 genes homólogos a *GCN20*, 5 de ellos pertenecen a un grupo claramente diferenciado, cuyo homólogo más cercano en levadura es la proteína GCN20 (Sanchéz-Fernandez et al., 2001). Uno de esos genes es *SCORD5 "Susceptivle to Coronatine (COR)-Deficient".* Fue identificado durante la búsqueda de un mutante de *Arabidopsis* que pudiera rescatar la virulencia producida por una bacteria mutante deficiente en COR, toxina producida por la bacteria *Pseudomonas syringae* (Zeng et al., 2011). Recientemente se ha demostrado que SCORD/GCN20 interactúa con GCN1 en *Arabidopsis*, sin embargo, no es necesaria para la fosforilación de eIF2α (Izquierdo et al., 2018).

Los fenotipos presentes en los mutantes *scord5/gcn20*, presentan fenotipos similares a los mutantes *ila-1* e *ila-2*. Estos mutantes muestras hojas amarillentas y no pueden cerrar los estomas después de la infección bacteriana (Zeng et al., 2011). Además, son

sensibles al ácido bórico y a la anticimicina A (Izquierdo et at, 2018). Por otro lado, tanto GCN2, GCN1 como GCN20 son susceptibles al inhibidor de la síntesis de aminoácidos CHL, pero solo GCN2 y GCN20 son sensibles a la paromomicina. Además se ha demostrado que GCN20 está implicado en el desarrollo de raíces (Tong-Tong Han et al., 2018).

2.OBJETIVOS

2. OBJETIVOS

El objetivo general de esta tesis doctoral es estudiar el papel de las proteínas GCN2, GCN1 y GCN20 en *Arabidopsis thaliana* y estimar así la relevancia de este sistema en la respuesta general al estrés ambiental en plantas. Para lograr este objetivo general nuestros objetivos específicos son:

1. Obtener o generar líneas mutantes de pérdida de función de: GCN2 *(gcn2-1, gcn2-2)*, GCN1 *(ila3)* y GCN20 *(gcn20/scord5)*. De estas líneas, se pretende obtener los siguientes resultados:
    1. Estimar su capacidad para fosforilar o no el factor de iniciación eIF2α.
    2. Caracterizar su fenotipo en condiciones normales y en condiciones de estrés abiótico.
    3. Estimar las causas moleculares de los fenotipos observados mediante experimentos transcriptómicos entre los diferentes mutantes y diversos análisis fisiológicos y moleculares.

2. Clonar los genes o dominios necesarios para realizar estudios de interacción molecular.

3.ARTÍCULOS

3.1.ARTÍCULO 1

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RESEARCH ARTICLE Open Access

Protein kinase GCN2 mediates responses to glyphosate in *Arabidopsis*

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Protein kinase GCN2 mediates responses to glyphosate in *Arabidopsis*.

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Keywords: Glyphosate, Gcn2, transcriptomic, shikimate, translation, herbicide

Abstract

BACKGROUND: The increased selection pressure of the herbicide glyphosate has played a role in the evolution of glyphosate-resistance in weedy species, an issue that is becoming a threat to global agriculture. The molecular components involved in the non-target-based resistance to this herbicide are still unidentified.

RESULTS: In this study, we identify the protein kinase GCN2 as a cellular component that fosters the action of glyphosate in the model plant *Arabidopsis thaliana*. Comparative studies using wild-type and *gcn2* knock-out mutant seedlings show that the molecular programme that the plant deploys after the treatment with the herbicide, is compromised in *gcn2*. Moreover, *gcn2* adult plants show a lower inhibition of photosynthesis, and both seedlings and adult *gcn2* plants accumulate less shikimic acid than wild-type after treatment with glyphosate.

CONCLUSIONS: These results points to an unknown GCN2-dependent factor involved in the cascade of events triggered by glyphosate in plants. Data suggest either that the herbicide does not equally reach the target-enzyme in a *gcn2* background, or that a decreased flux in the shikimate pathway in a *gcn2* plants minimize the impact of enzyme inhibition.

Background

Since its introduction in 1974, glyphosate has become the world most widely used herbicide, especially after the emergence of transgenic resistant crops in 1996. In 2007, more than 80% of the transgenic crops worldwide were engineered to be glyphosate-resistant [1]. An increase in the application frequency of the herbicide has, however, played a role in the evolution of glyphosate-resistance in weedy species, an issue that is becoming a threat to global agriculture. At least three of the ten most conspicuous weeds have evolved resistant to glyphosate after one decade of transgenic crops [2]. Therefore, it seems urgent to understand the molecular basis of resistance in order to safeguard the future use of glyphosate as an herbicide [1].

Resistance to herbicides can be achieved by alterations in the gene encoding the target protein, causing a reduction in the efficacy of the herbicide (target-site resistance) or by any other mechanism independent of the target enzyme (non-target-site resistance, NTSR) [3]. NTRS, that includes decreased herbicide penetration into the plant, decreased rate of herbicide translocation and increased rate of herbicide sequestration/ metabolism, has been reported to be the most widespread type of resistance to glyphosate [4]. Although several of these mechanisms have been proposed [5,6], the molecular components involved are still unidentified.

Glyphosate affects plants systemically after application to the leaf surface. The phytotoxic symptoms develop slowly, plant death requiring days or weeks depending on the dose applied [7]. Inhibition of its target enzyme 5-enolpyruvylshikimate-3-phosphate-synthase (EPSPs; EC 2.5.1.19), inhibits the shikimate pathway, leading to a shortage in aromatic amino acids, quinones and cofactor biosynthesis. This is considered by some authors as the main cause of glyphosate toxicity, consistent with the slow development of symptoms [7]. In contrast, others consider that shikimate accumulation, due to a decrease in feedback inhibition through the pathway, leads to an energy drain imposed by a utilization of one phosphoenolpyruvate (PEP) molecule and one ATP molecule for every molecule of shikimate-3-phosphate accumulated and 3 ATP's for every NADPH [8,9]. Herbicides inhibiting amino acid biosynthesis also induce non-target indirect effects, as proteolysis and an increase in free amino acids [10]. The last omics studies [11, 12] reveal that the full picture of molecular disturbances after EPSP inhibition is complex and far from being totally understood. One of the biochemically best characterized cellular events after glyphosate treatment is the rapid shutdown of photosynthesis [13]. Cessa-

tion of carbon fixation, decrease in chlorophyll content and electron transport has been reported to occur soon after herbicide application. This impairment is not only a metabolic perturbation but involves gene expression and protein contents. Turfgrass and soybean plants exposed to glyphosate repressed most genes related to photosynthesis [14, 15] and repression of photosynthetic proteins was observed in rice after glyphosate treatment [11]. Besides photosynthesis, other cellular processes such as cell cycle [16] cell motility (in bacteria) [12], cell death and redox homeostasis [11] are directly or indirectly affected by glyphosate.

In yeast, amino acid starvation is followed by activation of the protein kinase GCN2 by uncharged tRNAs. This enzyme phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α), inhibiting the conversion of eIF2γ-GDP to eIF2γ -GTP, preventing further cycles of translation initiation and suppressing protein synthesis [17]. Phosphorylation of eIF2α not only causes a general reduction of protein synthesis, but initiates the selective translation of GCN4, an mRNA containing short open reading frames (ORF) upstream of the long, protein-coding ORF. Its translation upon amino acid starvation produces a transcription factor that activates amino acid biosynthesis genes, helping the cell to recover from the stress. This regulatory response is known as general amino acids control (GAAC) [18].

Plants contain a GCN2 homologue kinase that complements the *gcn2* yeast mutant strain, indicating that the GAAC response could be also operating in plants [19]. In *Arabidopsis* plants with the aromatic amino acids biosynthesis impaired as imposed by glyphosate treatment, eIF2α is phosphorylated, and this phosphorylation is GCN2-dependent, as it was abolished in an insertion line in the *GCN2* gene [20]. Phosphorylation of eIF2α by GCN2 has been observed under several abiotic stresses [20, 21], and a GCN2-dependent translational arrest has been observed after treatment with chlorsulfuron, an inhibitor of branched amino acid biosynthesis [21]. Despite these evidences, the importance of GCN2 pathway as a regulatory mechanism in plants is still under debate [22-24]. The potential homologous gene of the yeast GCN4 has not been found in *Arabidopsis*, and little evidence was found for the involvement of AtGCN2 in the regulation of expression of amino acid biosynthesis genes, suggesting that this kinase could be playing another role than just regulating amino acid biosynthesis [25]. The role of GCN2 in the plant response to glyphosate remains elusive.

The transcriptional response to glyphosate has been determined in the bacteria *Escherichia coli* [12] in the grass *Festuca* [14] and in soybean [15] and in all cases a large number of genes change in expression in response to the herbicide. In the model plant *A. thaliana*, however, only a few genes (sixteen) were detected as modulated by glyphosate in leaves [26]. This discrepancy, and the unexpected behavior of aAtGCN2 in the regulation of amino acid biosynthetic genes, prompted us to reinvestigate the transcriptional response to glyphosate in *Arabidopsis* and to give some insights into the role of GCN2 in the triggering of this response.

In this study we show that glyphosate treatment triggers a complex transcriptional response in *Arabidopsis* plants. Surprisingly, many of these responses are not triggered or are altered in a *gcn2* mutant line. We also show that shikimate accumulation in *gcn2* plants after herbicide treatment is lower than in wild-type plants. All these results indicate that GCN2 is an important factor in the response of plants to glyphosate and that this protein kinase fosters the action of the herbicide by some unknown mechanism.

Results

Glyphosate treatment causes a dramatic rearrangement of the *Arabidopsis* transcriptome.

To get a better understanding of the transcriptional changes that glyphosate treatment provoke in *Arabidopsis* plants, 16-day-old plantlets were submerged in 200 μM glyphosate for 1 min, and gene expression was analysed 6 h after treatment. None of the glyphosate-derived phenotypic effects in the plant were visible at this time. Compared to mock-treated plants of the same age, more than 200 gene ontology (GO) biological processes were altered with a threshold applied of 0.05 (adjusted p-value, materials and methods). As expected, GO categories such as response to drug (adj. p-value 0.004) or multidrug transport (adj. p-value 0.003) are enriched in the glyphosate treated sample. In fact, 10 out the 30 most induced genes are ABC transporters, glutathione-transferases or glycosyltransferases [see additional file 1], enzymes known to be involved in the detoxification of herbicides [27]. Also expected, *Arabidopsis* plants respond to glyphosate activating the metabolism of aromatic amino acids (AAA) ($0.1x10^{-5}$), including some genes in the biosynthetic pathways, but also genes of the secondary metabolism pathways that have AAAs as precursors (such as lignin, auxins, phenylpropanoid and oth-

ers). For instance, genes coding for arogenate dehydratase, which catalyze the last step of phenylalanine (Phe) biosynthesis, and anthranilate synthase, phosphoribosylanthranilate transferase, indole-3-glycerol-phosphate synthase and trypthophan synthase, involved in trypthophan (Trp) biosynthesis, are all induced by glyphosate. Moreover, genes coding for phenylalanine-ammonia-lyase, (involved in phenylpropanoids biosynthesis, using Phe as a precursor), CYP79B3, or CYP71B15, (involved in IAA and camalexin biosynthesis, respectively, both using Trp as a precursor) are also induced. In addition, a plethora of genes belonging to many defense-related categories, including wounding, heat, oxidative, osmotic, cold, and biotic stresses are also induced, suggesting that not only specific, but also general responses, are triggered by a particular stress. Finally, categories such as aging or programmed cell death are enriched among the induced genes, revealing that, as early as 6 h after herbicide treatment, the plant could be already committed to die (see Table 1 and additional file 3).

Most remarkable, the herbicide-treated plants show a dramatic down-regulation of the photosynthesis ($7.06x10^{-29}$ adj. p-value), including chlorophyll biosynthesis ($3.89x10^{-07}$), electron transport chain ($1.26x10^{-08}$), and, to a lesser extent, CO_2 fixation ($4.68x10^{-03}$) (Table 1 and additional tables 2 and 4), a fact that was already observed at a later time-point (5 days after treatment) by Cebeci and Budak, [14] in turtgrass. Interestingly, categories related to translation, growth and cell division, are also down-regulated [see additional file 4], probably indicating that plant metabolism is being reprogrammed to cope with the stress situation [28]. The same down-regulation accounts for transcripts involved in redox homeostasis.

The presence of the protein kinase Gcn2 is necessary for the deployment of early cellular responses after glyphosate treatment.

GCN2 is a protein kinase that phosphorylates the α subunit of the eIF2 translation initiation factor, a key regulatory mechanism that arrest general protein synthesis and allows the re-stablishment of homeostasis in eukaryotes after several stress conditions [17]. In *Saccharomyces cerevisiae*, GCN2 is activated under amino acid starvation and triggers a general translational arrest but also promotes the selective translation of the transcription factor GCN4. This factor activates transcription of several hundred genes, including those involved in amino acid biosynthesis [17]. As glyphosate is known to block AAA biosynthesis, we wanted to know whether a similar mechanism was operating in plants, and also to identify other cellular responses to glyphosate eventually regulated by GCN2.

We compared the transcriptome of 16-day-old *Arabidopsis* wild-type seedlings with that of *gcn2* mutant seedlings of the same age [20], both at 6 h after glyphosate treatment. As described in Zhang et al 2008, *gcn2* plants are phenotypically indistinguishable from wild-type plans at this and all stages of growth. Previously, we had compared the transcriptome of the same plants under normal conditions and determined that only 24 genes were changing their expression using the selected criteria. [see additional files 1 and 2]. As shown in Additional Figure 1, and as reported by Zhang et al [20], eIF2α phosphorylation indicates that, after 6 h treatment, GCN2 kinase is activated in wild-type plants.

Interestingly, more than a thousand genes are differentially expressed in the *gcn2* mutant line after treatment with glyphosate as compared with the wild-type, according to the criteria specified in materials and methods. Looking at more detail, around 75% of the genes regulated by glyphosate are regulated with the same trend in the *gcn2* plants, but being the fold-change lower in the mutant plants [see additional files 1 and 2]. That is, a gene induced by glyphosate can still be induced in a *gcn2* mutant, but with a lower fold-change. This effect is more remarkable for those genes with the highest levels of expression. This observation suggests that cellular responses to glyphosate are compromised although not totally abolished in the *gcn2* mutant. The transcriptional repression of the photosynthesis is the biological process most affected by the lack of GCN2, as stated by GO biological processes studies [see additional file 5 and Table 2]: the expression of genes belonging to photosynthesis processes is significantly higher in *gcn2* plants after glyphosate treatment ($1.59x10^{-25}$ adjusted p-value, Fig2b), indicating that the repression that takes place in wild-type after glyphosate treatment is also compromised. The same effect is observed for cell division, redox homeostasis and other categories. Equally, among the genes previously shown to be induced by glyphosate that are deregulated in the *gcn2* mutant line are those involved in defense against both biotic and abiotic stimulus. As reported by Zhang et al [20], our functional categories data show that amino acid biosynthesis is not differentially regulated between wild-type and *gcn2* plants (supplemental table 4), although differences in expression were found for specific genes of the pathway (see supplemental table 1 and 2).

gcn2 plants show less glyphosate-derived effects and are partially resistant to the herbicide.

The observed dependence on the GCN2 kinase in glyphosate-induced responses was confirmed in adult plants. 200 μM glyphosate was sprayed on four-week-old *Arabidopsis* wild-type and *gcn2* plants and growth over the next three weeks after treatment was followed. Again, *gcn2* plants are phenotypically indistinguishable from wild-type plans at this stage of growth before the treatment. As observed in Fig. 1A and B, wild-type plants start showing typical phytotoxic glyphosate effects after 12-14 days. Meristems and young tissues became chlorotic and plant died in the following weeks. In less-affected plants, meristems looked disorganized, apical dominance was lost and plants presented a characteristic shoot-branching phenotype. Interestingly, these effects were not observed in *gcn2* plants, or they were much less severe. Although a subtle sensitivity could be observed the first days after treatment (initial chlorosis at the meristem area, data not shown), we did not observe glyphosate effects in *gcn2* plants two-weeks after treatment, when chlorotic tissues were clearly observed in wild-type plants. Much later, whereas wild-type plants were dying, *gcn2* plants recovered from the stress and were growing normally (data not shown)

To evaluate in more detail the biochemical differences between both genotypes, the effects of glyphosate on various parameters associated with photosynthetic was measured in non-chlorotic healthy leaves two-weeks after treatment, when the first symptoms of the herbicide were already visible in younger tissues. As shown in Table 3 and described previously in other works [29 and references therein], photosynthetic rate, stomatal conductance, transpiration and quantum efficiency of photosystem II were rapidly inhibited in Ler-0 leaves sprayed with glyphosate as compared with mock-treated plants. The increase in substomatal CO_2 concentration with decreasing stomatal conductance suggests biochemical limitations to photosynthesis. Although no changes in Fv/Fm were observed, the herbicide provoked a significant decrease in chlorophyll content.

By contrast, the application of the herbicide has negligible effects on photosynthetic measurements in the *gcn2* mutant plants. No significant differences were observed between mock-treated and glyphosate-treated plants on CO_2 assimilation, stomatal conductance, quantum efficiency of photosystem II or maximal quantum yield (Table 3). These results further confirm the dependence on GCN2 of the glyphosate-induced repression of photosynthesis.

These results agree with the relative expression of several photosynthetic genes in both genotypes. We analyzed by RT-PCR the expression of the three genes most repressed by

glyphosate in the seedlings experiment, namely two light-harvesting complex genes (LHC 2.2 and LHC 4.2) and a gene involved in the assembly of cytochrome b_6f (At5g36120). These three genes were not repressed in *gcn2* seedlings [see additional file 2]. As shown in Fig. 1.D, expression of these genes was repressed in wild-type plants after glyphosate treatment, but the effect was not observed in the *gcn2*-treated plants, confirming the results obtained in seedlings.

DAB staining is used as an efficient method to detect hydroxen peroxide accumulation in plant tissues [30] and H_2O_2 increases have been described in plants treated with glyphosate [9]. Young leaves of wild-type plants after glyphosate treatment were stained using DAB. As shown in Fig 1.C, a dramatic accumulation of H_2O_2 is observed, preceeding the cell death observed in the following days. As expected, *gcn2* plants of the same age did not show H_2O_2 accumulation, further confirming the dependence on GCN2 of the oxidative-stress burst observed after glyphosate treatment.

In summary, gene expression data, as well as glyphosate effects such as photosynthesis decay and oxidative stress, suggest that *gcn2* plants are less affected by glyphosate than wild-type plants.

Shikimic acid accumulation is compromised in *gcn2* plants after glyphosate treatment.

Glyphosate inhibition of the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase leads to reduced feedback inhibition of the pathway, resulting in carbon flow to shikimate-3-phosphate, which is converted into high levels of shikimate [7]. Given that *gcn2* plants were less prone to glyphosate effects, as observed in the experiments described above, we wanted to determine if the target enzyme of glyphosate was inhibited in the same way in both lines. *Arabidopsis* wild-type and *gcn2* seedlings were treated with glyphosate as explained above, and shikimic acid accumulation was measured three days after treatment. The same experiment was performed in four-weeks old plants, in the way explained above and in Materials and Methods. As observed in Fig 2, basal levels of shikimic acid are similar in both lines, either in seedlings or in adult plants. However, 3-days after glyphosate application, shikimic acid in wild-type seedlings has increased almost 20 times over the basal levels (46 times in adult plants treatments), whereas *gcn2* only accumulates half the amount of shikimic acid that wild-type does (only 10 times in seedlings, 20 times in adult plants). This trend indicates that the target enzyme of

glyphosate is not being inactivated at the same extend in *gcn2* plants, or that the metabolic flux to the pathway has been reduced in *gcn2* plants.

Glyphosate uptake is not affected in *gcn2* plants

In the plant, EPSPS is encoded in the nucleus and translocated to the chloroplast, where the aromatic amino acids are synthesized [31]. The highest shikimate accumulation occurs in the youngest tissues, suggesting that glyphosate enters the plant and then translocates to the active meristems to reach the target site, where its action takes place preferentially [7]. Several reasons could then account for the less glyphosate action observed in *gcn2* plants. In one hand, glyphosate uptake and/or translocation could be compromised. Less concentration of active glyphosate will be present in the plant chloroplasts, explaining the apparent resistance of *gcn2* plants to the herbicide. On the other hand, detoxification mechanisms could be exacerbated in the mutant, inactivating the herbicide.

The mechanisms of glyphosate uptake into plant cells are not well understood. At least at low concentrations of herbicide (in the micromolar range, as it is used in this study) it seems to involve a phosphate transporter, as glyphosate uptake is inhibited by sodium phosphate and phosphonoformic acid, a competitor inhibitor of phosphate transport in plants [32, 33]. In order to ascertain if *gcn2* plants were compromised in phosphate transport, that could confer to these plants an advantage in the presence of the herbicide, we performed germination assays of wild-type and *gcn2* seeds in media with phosphate deficiency. No differences were observed between wild-type and *gcn2* seedling when grown in media containing 0-500μM phosphate (data not shown), indicating that *gcn2* plants are taking up phosphate in a similar way as wild-type does. The same results were observed in root-growth assays (data not shown).

Discussion

Little is known about the molecular events that contribute to non-target-site based resistance (NTSR), the combination of mechanisms that limit to a non-lethal dose the amount of herbicide reaching the target-site. In this study, we identify GCN2 as a cellular component that fosters the action of glyphosate in the model plant *A. thaliana*. GCN2 is a conserved protein kinase responsible for the phosphorylation of the initiation

factor eIF2-α after a number of stress situations. In *Arabidopsis*, herbicide treatments, wounding, cold treatments, UV or purine starvation has been described to activate AtGCN2 [21] the only kinase able to phosphorylate eIF2-α in this species [20]. Phosphorylation of eIF2-α prevents further cycles of protein translation, and it is assumed that this helps the cell to conserve metabolic resources until it has overcome the immediate biological impact of the stress [28]. In this model, the activation of GCN2 should then be beneficial for the plant to cope with the stress. However, we have shown that the presence of GCN2 is somehow facilitating the action of the herbicide, and cellular responses to glyphosate are not triggered or attenuated in a mutant line that is not able to phosphorylate eIF2-α. This is not the first report where the lack of GCN2 is conferring an advantage when a particular stress is applied. In yeast, ScGCN2 acts also as a negative factor, conferring toxic effects on growth under NaCl stress, and being a *gcn2* knock-out strain able to grow normally under 400mM NaCl [34]. In human tumor cells, HsGcn2 was shown to have an unexpected proapoptotic effect under glucose deficiency stress, and *gcn2* knock-out cells are able to survive more than wild-type under these stress conditions [35]. As such, these stresses and the canonical amino acid starvation may utilize distinct pathways that converge on eIF2α phosphorylation with opposing biological outcomes [35]. It will be interesting to know whether a *gcn2* background confers resistance to other abiotic stresses in *Arabidopsis*, and to investigate if this resistance is converging in a single factor for all stresses or in different factors for every one of them.

Known responses to glyphosate include the rapid repression of photosynthesis. Inhibition of CO_2 assimilation and depletion of intermediates of the carbon reduction cycle had been documented years ago [36] and these effects had been attributed to an upregulated flux into the shikimate pathway due to depletion downstream of EPSPs. Recent transcriptomic assays revealed that this repression could be also genetically regulated. In a comparative study of *Festuca* species, Cebeci and Budak reported that, 5 days after treatment with glyphosate, a marked repression of photosynthetic genes, including chlorophyll biosynthesis, photosystems and Calvin cycle enzymes was occurring [14]. Consistent results were obtained in proteomic assays performed in rice [11]. We have also observed repression in gene expression in adult plants 15 days after treatment, consistent with a clear inhibition of photosynthetic rate. The dramatic repression of photosynthetic genes only 6 h after treatment to *Arabidopsis* seedlings, when no visible symptoms of leaf chlorosis were observed yet (Table 1), suggests that besides a likely photosynthetic decrease due to metabolic toxicity, an early genetically programmed inhibition

of photosynthesis is also taking place. Consistent with our data, repression in some photosynthetic genes was observed in soybean sensitive plants only 4 h after treatment with glyphosate [15]. This decay of photosynthesis after glyphosate treatment is not observed in *gcn2* plants, nor is the oxidative stress that characterizes the herbicide effect (Table 2). Moreover, enzymes known to be involved in the detoxification of herbicides such as ABC transporters, glutathione-transferases or glycosyltransferases [37,38] are dramatically activated after glyphosate treatment in wild-type plants, but unaltered or weakly activated in *gcn2* plants [see additional file 1]. Finally, shikimate accumulation in *gcn2* plants compared to wild-type clearly demonstrates that the lack of GCN2 becomes an advantage when plants are treated with this herbicide.

At present, these data do not allow to discern at what point the herbicide action benefits from the presence of GCN2 in the cell. Glyphosate is said to starve cells of aromatic amino acids, due to inhibition of the shikimate pathway and the production of chorismate, the precursor of phenylalanine, tryptophan and threonine. However, total amino acid pools after glyphosate treatment rather increase than decrease [39, 40] after the first days after treatment, as a consequence of the induction of some proteolytic activities [10] or are not much different in glyphosate resistant soybean as compared to sensitive cultivars [29]. The known mechanism of activation of GCN2 through uncharged t-RNAs [18] also works in *Arabidopsis* [41] and although not described in detail for glyphosate, treatment with chlorsulfuron, that blocks valine, leucine and isoleucine biosynthesis [20] yield a peak of eIF2-α phosphorylation 6 h after treatment but return to basal levels after 24h. Starvation of amino acids and prolonged protein translational arrest via GCN2 are then unlikely to be the major cause of the slow effects of glyphosate treatment. The involvement of GCN2 in the glyphosate mode of action should then fall on the first hours after the treatment, this conditioning the final effect in the plant. How could GCN2 foster the action of the herbicide? As stated above, the early activation of the kinase in wild-type plants after glyphosate application, likely due to the initial decay in aromatic amino acids, do not avoid the expression of cellular factors involved in detoxification of xenobiotics. In some species, vacuolar sequestration is contributing to the resistance mechanism in resistant variants [42]. If the same mechanism is working in *Arabidopsis,* activation of GCN2 could impair vacuolar membrane trafficking through inhibition of some important protein. Alternatively, the selective translation of certain mRNAs with uORFs in the leader sequence, in the same way of *ScGCN4*, in yeast, and *HsATF4*, in humans [18], could facilitate translocation of glyphosate to the young tissues,

where the target enzyme is mainly expressed and the action of glyphosate is more dramatic [7]. Reduced translocation to meristematic sinks is a major mechanism of resistance in horseweed [43], Italian ryegrass [44] or hairy fleabane [45]. However, no homologous sequence of *ScGCN4* has been found in *Arabidopsis* so far, and a GCN2-dependent selective translation of mRNAs is unknown. Finally, the absence of GCN2 activity in the mutant *gcn2* line could provide a constitutive advantage in the mutant background that diminished herbicide effects, independently of the post-treatment GCN2 activation. One possibility was a higher uptake of glyphosate in wild-type plants. Although the mechanism of glyphosate uptake into plant cells is not well understood, the involvement of a phosphate transporter has been proposed [32, 46]. *gcn2* plants are not more sensitive than wild-type to growth in phosphate deficiency media, and microarray experiments over non-treated seedlings [see additional file 1] did not reveal differences in gene expression that make suspect of phosphate transport missregulation, indicating that phosphate transport is not compromised in *gcn2* plants. If phosphate transporters are involved in glyphosate uptake in *Arabidopsis*, then *gcn2* plants should be taking up the herbicide at the same rate than wild-type plants.

Several attempts have been made to find genes involved in glyphosate resistance using mutant collections of *A. thaliana* [47, 48]. In Brotherton et al., the same concentration of glyphosate used in this study was used in a germination assay to find EMS-mutagenized mutant lines of *Arabidopsis* resistant to the herbicide, but no resistant mutant was recovered [48]. If glyphosate resistance single mutations were common, they should have been found in these saturation mutagenesis studies [47]. The lack of *GCN2* does not confer resistance to glyphosate in germination assays (data not shown). In seedling, the *gcn2* mutant line showed sensitivity to glyphosate treatment [20]. However, we have shown that shikimate is not accumulating and gene activation is not being triggered at the same rate than in wild-type plants, indicating that this protein kinase could be an important clue to discover components involved in the non-target based resistance to this herbicide. “Glyphosate is as important to world agriculture as penicillin is to human health” stated Stephen Powles, director of the Australian Herbicide Resistance Initiative (http://www.ahri.uwa.edu.au). Given the spread of glyphosate resistance weeds around the planet and the economic importance for agriculture, understanding the mechanisms of such resistance could help to design new biotechnological approaches for a more efficient use of this important herbicide.

Conclusions

Several mechanisms have been proposed for non-target based resistance to glyphosate, but any study has so far identified any gene that could be directly involved or influencing the final effect of this herbicide in the plant. We have demonstrated that the translational regulator GCN2 is fostering the action of the herbicide by an unknown mechanism. The loss-of-function *gcn2* mutant in the model plant *A. thaliana* emerge as an important tool to decipher the way glyphosate enters the plant and reach its target site. This information will help to design new strategies to preserve the use of glyphosate in the emerging glyphosate-resistance-weeds era.

Methods

Plant Growth and Treatments

A. thaliana accession Landsberg erecta (Ler-0) was used in this study. Genetrap GT8359, containing a Ds transposable element interrupting the *GCN2* gene [20] was obtained from Cold Spring Harbor Laboratory, New York (http://genetrap.cshl.org).

Seeds were pretreated in 70% ethanol for 20 min, surface-sterilized in 2.5% bleach for 10 min, and washed with distillated water at least five times. After stratification at 4°C in the dark for 5 days, seeds were sown on 1% agar-containing MS Salts, 1% sucrose, pH 5.5, and grown at 23°C with a 16-h-light/8-h-dark cycle.

For experiments at seedling stage, 16-day-old plantlets were submerged in 200 μM glyphosate (SIGMA) or distilled water (mock) for 1 minute, and incubated for further growth on liquid MS medium, 1% sucrose, pH 5.5, under the same conditions. Samples for microarray experiments were collected 6 h after treatment. Samples for shikimate assays were collected 72 h after treatment. Visual inspection was observed during the next two weeks after treatment.

For experiments in adult plants, 10 to 15-day-old plantlets were transferred to soil and grown at 23ºC under short-day conditions (8-h-light/16-h-dark). Glyphosate treatments were done three-weeks later. Plants were sprayed with 200 μM glyphosate once (or distilled water for mock treatments), and incubated for further growth under the same conditions. Expression analysis by RT-PCR, DAB staining and photosynthetic measure-

ments were done two-weeks after treatment. Visual inspection was followed during the next four weeks after treatment.

Microarray Experiments

Total RNA of glyphosate- and mock-treated 16-day-old seedlings was extracted using RNeasy kit (Qiagen). 1.5 µg of total RNA was labeled using MessageAmp II amplification kit from Life Technologies, following manufacturer instructions. Labeling was done using Cy3 and Cy5 dyes from GE (RPN5661). Before hybridization, slides were pre-hybridized at 42ºC for 45 m in 5xSSC, 0.1%SDS and 0.1 mg/mL BSA. Microarray hybridizations and washings were done in manual chambers at 42ºC, according to Forment et al. [49]. Scanning and Image Analysis was performed using GenePix Pro 6.0 software (Molecular Devices).

For wild type vs. wild type + Glyphosate, two biological replicates were done. Expression ratios in both microarrays were averaged and considered for further analysis if equal trend (induction or repression) was observed in both replicates. Gene set Enrichment Analysis was done using Fatiscan [50] taking as significant those categories with and adjusted p-value lower than 0.05.

For *gcn2* mutant + Glyphosate vs. wild type + Glyphosate, three biological replicated were done swapping the dyes in one of them. A gene was considered differentially expressed if average fold-change was higher than 2 and had a FDR<5% after a SAM test [51]. Functional analysis was done using FatiGO [49] taking as significant those categories with and adjusted p-value lower than 0.05. The same analysis was performed for *gcn2* mutant vs. wild type without glyphosate.

These microarrays data have been included in the GEO Omnibus database with the reference numbers GSE 56146 and GSE 56147.

Shikimate assay

Shikimate determination was done 3 days after treatment in seedlings and 7 days after treatment in adult plants. For the seedling assay, fresh weight was annotated before freezing. From each adult leaf, three discs (4 mm diameter using a micropunch) were placed in a 2-ml tube. Seedlings and discs were frozen with liquid nitrogen and kept at -80 until use. Extraction of shikimate was performed as described in Koger et al [52]. Vials were removed from the freezer and 0.25 M HCl was added to each vial (1 ml per

100 mg FW or 100 µl per leaf disc). Vials were mixed by vortexing and incubated at room temperature for 1.5 h. Afterwards the solution was frozen and kept at -20ºC until analysis. Shikimate was analyzed by HPLC as described before [39].

Photosynthetic measurements

Gas exchange and chlorophyll fluorescence measurements were performed as described by Flexas et al. [53]. 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 with a LI-6400 (LICOR, Nebraska, USA). 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}) [54]. 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 on the 8th to 10th leaf from the apex, and for each genotype and treatment, 8 different plants were measured.

DAB staining

In situ detection of hydrogen peroxide was performed by staining with diaminobenzidine (DAB) staining, according to Daudi et al [30] with modifications. Briefly, rosette leaves were incubated in staining buffer (1 mg/mL DAB containing Tween 20 (0.05% v/v) and 50 mM sodium phosphate buffer (pH 3.8) and vacuum infiltrated applying 3 pulses of 1.5m, and stained for 24h at room temperature. Leaves were fixed in ethanol:glycerol:acetic acid 3:1:1 (bleaching solution) placed in a water bath at 95°C for 15 m. Bleaching solution was replaced and plants were visualized under white light and photographed.

Real-time PCR

For RT-PCR experiments, total RNA was extracted using RNeasy kit (Qiagen) and treated with DNase I to remove genomic DNA. cDNA was obtained using the Maxima First Strand cDNA Synthesis Kit (Fermentas). Quantitative real-time PCR was performed in a 7500 Fast Real-Time PCR System, from Applied Biosystems, using EvaGreen as a fluorescent

reporter and Taq polymerase (Biotools). Primers were designed using PRIMER3 software. Actin 8 (At1g49240) was used as an internal control (Fw 5'-AGTGGTCGTACAACCG-GTATTGT; Rv 5'- GAGGATAGCATGTGGAAGTGAGAA). Primers for LHCB 4.2 (Fw 5'-CCACTCTTGGCGCTATCAC; Rv 5'- GCCGATCACTAACACTTCGAT). Primers for LHCB 2.4 (Fw 5'- AGCGACCTCATCCAAAAGG; Rv 5'- TCCGAGAATGGTCCCAAGTA). Primers for B6F (Fw 5'-AGTGACCACCAGCTTCGTCT; Rv 5'- AAGAGACGTGGATCGATTGC) The reaction commenced at 95°C for 5 m, followed by 40 cycles of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Data were analyzed using 7500 Applied Biosystem proprietary software v.2.0.4.

Availability of supporting data

The data sets supporting the results of this article are available in the GEO repository (GSE56146 and GSE56147).

Authors' contributions.

IF performed the RT-PCR experiments, prepared plants for all experiments, performed the DAB experiments and contributed to microarrays assays. SN carried out the photosynthetic measurements. AZ and MR performed the shikimate assays. JS performed phenotypic analysis and helped in data interpretation. RS revised the manuscript and gave important intellectual contribution to the work. JG designed the experiments, performed microarray experiments and analysis, and write the manuscript.

Competing interests.

The authors declare no competing interests in this work.

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References

[1] James C. **Global status of Commercialized Biotech/GM Crops**: www.isaaa.org, 2010.

[2] Basu C, Halfhill MD, Mueller TC, Stewart CN Jr: **Weed genomics: new tools to understand weed biology**. *Trends Plant Sci*. 2004, **9(8):**391-8.

[3] Délye C. **Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade**. *Pest Manag Sci*. 2013, **69(2)**:176-87.

[4] Powles SB, Yu Q: **Evolution in action: plants resistant to herbicides**. *Annu Rev Plant Biol*. 2010, **61:**317-47.

[5] Ge X, d'Avignon DA, Ackerman JJ, Collavo A, Sattin M, Ostrander EL, Hall EL, Sammons RD, Preston C: **Vacuolar glyphosate-sequestration correlates with glyphosate resistance in ryegrass (Lolium spp.) from Australia, South America, and Europe: a 31P NMR investigation**. *J Agric Food Chem*. 2012, 60(5):1243-50.

[6] Ge X, d'Avignon DA, Ackerman JJ, Sammons RD. **Rapid vacuolar sequestration: the horseweed glyphosate resistance mechanism**. *Pest Manag Sci*. 2010 Apr;66(4):345-8

[7] Duke SO, Powles SB: **Glyphosate: a once-in-a-century herbicide.** *Pest Manag Sci*. 2008, **64(4):**319-25.

[8] Fisher RS, Berry A, Gaines CG, Jensen RA: **Comparative action of glyphosate as a trigger of energy drain in Eubacteria**. *J. Bacteriol* 1986, **168:**1147–1154.

[9] De María N, Becerril JM, García-Plazaola JI, Hernández A, De Felipe MR, Fernández-Pascual M. **New insights on glyphosate mode of action in nodular metabolism: Role of shikimate accumulation.** *J Agric Food Chem* 2006, **54**: 2621-2628

[10] Zulet A, Gil-Monreal M, Villamor JG, Zabalza A, van der Hoorn RAL, Royuela M:. **Proteolytic Pathways Induced by Herbicides That Inhibit Amino Acid Biosynthesis.** *PLoS ONE* 2013, **8(9)**: e73847.

[11] Ahsan N, Lee DG, Lee KW, Alam I, Lee SH, Bahk JD, Lee BH: **Glyphosate-induced oxidative stress in rice leaves revealed by proteomic approach.** *Plant Physiol Biochem* 2008, **46(12):**1062-70.

[12] Lu W, Li L, Chen M, Zhou Z, Zhang W, Ping S, Yan Y, Wang J, Lin M: **Genome-wide transcriptional responses of Escherichia coli to glyphosate, a potent inhibitor of the shikimate pathway enzyme 5-enolpyruvylshikimate-3-phosphate synthase.** *Mol Biosyst*. 2013, **9(3):**522-30.

[13] Servaites JC, Tucci MA, Geiger DR: **Glyphosate effects on carbon assimilation, ribulose bisphosphate carboxylase activity, and metabolite levels in sugar beet leaves.** *Plant Physiol*. 1987, **85(2):**370-4.

[14] Cebeci O, Budak H: **Global Expression Patterns of Three Festuca Species Exposed to Different Doses of Glyphosate Using the Affymetrix GeneChip Wheat Genome Array.** *Comp Funct Genomics* 2009, 505701.

[15] Zhu J, Patzoldt WL, Shealy RT, Vodkin LO, Clough SJ, Tranel PJ: **Transcriptome response to glyphosate in sensitive and resistant soybean**. *J Agric Food Chem*. 2008, **13;56(15):**6355-63.

[16] Marc J, Mulner-Lorillon O, Bellé R: **Glyphosate-based pesticides affect cell cycle regulation**. *Biol Cell*. 2004, **96(3):**245-9.

[17] Wek RC, Jiang HY, Anthony TG: **Coping with stress: eIF2 kinases and translational control.** *Biochem Soc Trans*. 2006, **34:**7-11.

[18] Hinnebusch AG: **Translational regulation of GCN4 and the general amino acid control of yeast**. *Annu Rev Microbiol* 2005, **59**:407-50.

[19] Zhang Y, Dickinson JR, Paul MJ, Halford NG: **Molecular cloning of an *Arabidopsis* homologue of GCN2, a protein kinase involved in co-ordinated response to amino acid starvation**. *Planta*. 2003, **217(4):**668-75.

[20] Zhang Y, Wang Y, Kanyuka K, Parry MA, Powers SJ, Halford NG: **GCN2-dependent phosphorylation of eukaryotic translation initiation factor-2alpha in *Arabidopsis*.** *J Exp Bot*. 2008, **59(11)**:3131-41.

[21] Lageix S, Lanet E, Pouch-Pelissier MN, Espagnol MC, Robaglia C, Deragon JM, Pelissier T: ***Arabidopsis* eIF2a kinase GCN2 is essential for growth in stress conditions and is activated by wounding**. *BMC Plant Biol* 2008, **8,** 134–142.

[22] Shaikhin SM, Smailov SK, Lee AV, Kozhanov EV, Iskakov BK: **Interaction of wheat germ translation initiation factor 2 with GDP and GTP**. *Biochimie*. 1992, **74(5):**447-54.

[23] Krishna VM, Janaki N, Ramaiah KV: **Wheat germ initiation factor 2 (WG x eIF2) decreases the inhibition in protein synthesis and eIF2B activity of reticulocyte lysates mediated by eIF2alpha phosphorylation**. *Arch Biochem Biophys* 1997, **346(1):**28-36.

[24] Immanuel TM, Greenwood DR, MacDiarmid RM: **A critical review of translation initiation factor eIF2α kinases in plants-regulating protein synthesis during stress**. *Functional Plant Biology* 2012, **39:** 717-735

[25] Byrne EH, Prosser I, Muttucumaru N, Curtis TY, Wingler A, Powers S, Halford NG: **Overexpression of GCN2-type protein kinase in wheat has profound effects on free amino acid concentration and gene expression.** *Plant Biotechnol J*. 2012, **10(3):** 328-40.

[26] Das M, Reichman JR, Haberer G, Welzl G, Aceituno F, Mader MT, Watrud LS, Pfleeger TG, Gutierrez R, Schaffner AR, Olszyk DM: **A composite transcriptional signature differentiates responses towards closely related herbicides in *Arabidopsis thaliana* and *Brassica napus***. *Plant Mol Biol* 2010, **72:**545–556

[27] Yuan JS, Tranel PJ, Stewart CN Jr: **Non-target-site herbicide resistance: a family business**. *Trends Plant Sci*. 2007, **12(1):**6-13.

[28] Baena-González E: **Energy signaling in the regulation of gene expression during stress**. *Mol Plant*. 2010, 3(2):300-13.

[29] Vivancos PD, Driscoll SP, Bulman CA, Ying L, Emami K, Treumann A, Mauve C, Noctor G, Foyer CH: **Perturbations of amino acid metabolism associated with glyphosate-dependent inhibition of shikimic acid metabolism affect cellular redox homeostasis and alter the abundance of proteins involved in photosynthesis and photorespiration.** *Plant Physiol*. 2011, **157(1):**256-68.

[30] Daudi A, Cheng Z, O'Brien JA, Mammarella N, Khan S, Ausubel FM, Bolwell GP: **The apoplastic oxidative burst peroxidase in *Arabidopsis* is a major component of pattern-triggered immunity**. *Plant Cell*. 2012, **24(1):**275-87.

[31] Schmid J, Amrhein N.. **The shikimate pathway** 1999. In: Singh B, ed. Plant Amino Acids, Biochemistry and Biotechnology. Marcel Dekker, New York, 147–170.

[32] Denis, M-H, Delrot S: **Carrier-mediated uptake of glyphosate in broad bean (*Vicia faba*) via a phosphate transporter.** *Physiol. Plant* 1993, **87:**569–575.

[33] Hetherington PR, Marshall G, Kirkwood RC, Warner JM: **Absorption and efflux of glyphosate by cell suspensions.** *J. Exp. Bot* 1998, **49:**527-533.

[34] Goossens A, Dever TE, Pascual-Ahuir A, Serrano R: **The protein kinase Gcn2p mediates sodium toxicity in yeast**. *J Biol Chem* 2001, **276(33):**30753-60.

[35] Muaddi H, Majumder M, Peidis P, Papadakis AI, Holcik M, Scheuner D, Kaufman RJ, Hatzoglou M Koromilas AE: **Phosphorylation of eIF2α at serine 51 is an important determinant of cell survival and adaptation to glucose deficiency.** *Mol Biol Cell*. 2010, **21(18):**3220-31

[36] Geiger DR, Kapitan SW, Tucci MA: **Glyphosate inhibits photosynthesis and allocation of carbon to starch in sugar beet leaves.** *Plant Physiol* 1986, **82:**468-472

[37] Edwards R, Del Buono D, Fordham M, Skipsey M, Brazier M, Dixon DP, Cummings I: **Differential induction of glutathione transferases and glucosyltransferases in wheat, maize and *Arabidopsis thaliana* by herbicide safeners.** *Z Naturforsch*. 2005, **60(3-4):** 307-16.

[38] Cummins I, Wortley DJ, Sabbadin F, He Z, Coxon CR, Straker HE, sellars JD, Knight K, Edwards L, Hughes D, Kaundun SS, Hutchings S, Steel P, Edwards R. **Key role for a glutathione transferase in multiple-herbicide resistance in grass weeds.** *Proc Natl Acad Sci U S A*. 2013, **110(15):**5812-7.

[39] Orcaray L, Igal M, Marino D, Zabalza A, Royuela M: **The possible role of quinate in the mode of action of glyphosate and acetolactate synthase inhibitors.** *Pest Manag Sci*. 2010, **66:**262-269.

[40] Orcaray L, Zulet A, Zabalza A, Royuela M. **Impairment of carbon metabolism induced by the herbicide glyphosate**. *Journal of Plant Physiology* 2012 **169:** 27–33.

[41] Li MW, AuYeung WK, Lam HM: **The GCN2 homologue in *Arabidopsis thaliana* interacts with uncharged tRNA and uses *Arabidopsis* eIF2α molecules as direct substrates**. *Plant Biol (Stuttg)*. 2013, **15(1):**13-8.

[42] Ge X, Davignon DA, Ackerman JJ, Sammons D. **In Vivo 31P-NMR Studies of Glyphosate Uptake, Vacuolar Sequestration, and Tonoplast Pump Activity in Glyphosate Resistant Horseweed (Conyza canadensis)**. *Plant Physiol*. 2014 **Sep 2**. pp. 114.247197

[43] Feng PCC, Chiu T, Sammons RD: **Glyphosate efficacy is contributed by its tissue concentration and sensitivity in velvetleaf (Abutilon theophrasti).** *Pesticide Biochemistry and Physiology* 2003, **77:**83-91.

[44] Koger CH, Reddy KN: **Role of absorption and translocation in the mechanism of glyphosate resistance in horseweed (*Conyza canadensis*).** *Weed Sci*. 2005, **53:**84–89.

[45] Perez-Jones AKW, Park J, Colquhoun C, Mallory-Smith J, Shaner D: **Identification of glyphosate-resistant Italian ryegrass (Lolium multiflorum) in Oregon.** *Weed Science* 2005, **53:**775–779.

[46] Morin F, Vera V, Nurit F, Tissut M, Marigo G: **Glyphosate uptake in *Catharanthus roseus* cells: Role of a phosphate transporter.** *Pestic. Biochem. Physiol* 1997, **58:**13–22.

[47] Jander G, Baerson SR, Hudak JA, Gonzalez KA, Gruys KJ, Last R L: **Ethylmethanesulfonate saturation mutagenesis in *Arabidopsis* to determine frequency of herbicide resistance**. *Plant Physiol*; 2003, **131:**139–46.

[48] Brotherton JE, Jeschke MR, Tranel PJ, Widholm JM: **Identification of *Arabidopsis thaliana* variants with differential glyphosate responses.** *Journal of Plant Physiology* 2007, **164:**1337-1345.

[49] Forment J, Gadea J, Huerta L, Abizanda L, Agusti J, Alamar S, Andres F, Arribas R, Beltran JP, Berbel A, Blazquez MA, Brumos J, Canas LA, Cercos M, Colmenero-Flores JM, Conesa A, Estables B, Gandia M, Garcia-Martinez JL, Gimeno J, Gisbert A, Gomez G, Gonzalez-Candelas L, Granell A, Guerri J, Lafuente MT, Madueno F, Marcos JF, Marques MC, Martinez F, Martinez-Godoy MA, Miralles S, Moreno P, Navarro L, Pallas V, Perez-Amador MA, Perez-Valle J, Pons C, Rodrigo I, Rodriguez PL, Royo C, Serrano R, Soler G, Tadeo F, Talon M, Terol J, Trenor M, Vaello L, Vicente O, Vidal Ch, Zacarias L, Conejero V. **Development of a citrus genome-wide EST collection and cDNA microarray as resources for genomic studies**. *Plant Mol Biol*. 2005, **57(3):**375-91.

[50] Medina I, Carbonell J, Pulido L, Madeira SC, Goetz S, Conesa A, Tárraga J, Pascual-Montano A, Nogales-Cadenas R, Santoyo J, García F, Marbà M, Montaner D, Dopazo J: **Babelomics: an integrative platform for the analysis of transcriptomics, proteomics and genomic data with advanced functional profiling.** *Nucleic Acids Res*. 2010, Jul; 38

[51] Tusher VG, Tibshirani R, Chu G: **Significance analysis of microarrays applied to the ionizing radiation response.** *Proc Natl Acad Sci U S A*. 2001, **98(9):**5116-21.

[52] Koger CH, Shaner DL, Krutz LJ, Walker TW, Buehring N, Henry WB, Wilcut JW: **Rice (Oryza sativa) response to drift rates of glyphosate.** *Pest Manag Sci* 2005, **61(12):**1161-1167.

[53] Flexas J, Ortuño MF, Ribas-Carbo M, Diaz-Espejo A, Flórez-Sarasa ID, Medrano H: **Mesophyll conductance to CO_2 in *Arabidopsis thaliana***. *New Phytologist* 2007, **175:** 501–511.

[54] Genty B, Briantais JM, Baker NR: **The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence**. *Biochimica et Biophysica Acta*. 1989, **990:** 87–92.

Selected GO categories enriched in glyphosate-induced genes		Selected GO categories enriched in glyphosate –repressed genes	
GO biological process	**adj. P-value**	**GO biological process**	**adj. P-value**
proteolysis	$6.74x10^{-24}$	photosynthesis	$7.06x10^{-29}$
defense response	$5.60x10^{-12}$	microtubule-based movement	$3.71x10^{-9}$
response to wounding	$8.68x10^{-11}$	photosynthetic electron transport chain	$1.26x10^{-8}$
response to bacterium	$7.88x10^{-10}$	porphyrin biosynthetic process	$3.89x10^{-7}$
aromatic amino acid metabolic process	0.00000116 9	cell division	0.0000295
response to osmotic stress	0.0000400	electron transport chain	0.000260
cell death	0.001031	translation	0.00127
response to oxidative stress	0.00318	fixation of carbon dioxide	0.00468
multidrug transport	0.00373	regulation of cell size	0.00614
response to drug	0.00483	cell growth	0.011

Table 1. *Selected categories enriched in Arabidopsis seedlings after glyphosate treatment (for a complete list, see additional file 3)*

Selected categories of a Gene Set Enrichment Analysis using Fatiscan (Medina et al., 2010) on the expression values of 16-days-old *Arabidopsis* wild-type Landsberg erecta seedlings treated with glyphosate, as described in Materials and Methods. For a complete list of GO categories (biological process) with an adjusted p-value lower than 0.05, see additional file 3.

Selected GO categories enriched in wild-type as compared with gcn2 in glyphosate-induced genes		**Selected GO categories enriched in *gcn2* as compared with wild-type in glyphosate –repressed genes**	
GO biological process	**adj. p-value**	**GO biological process**	**adj. p-value**
response to wounding	$3.45x10^{-24}$	photosynthesis	$1.59x10^{-25}$
response to bacterium	0.0000377	microtubule-based movement	$8.52x10^{-12}$
autophagy	0.000157	chlorophyl biosynthetic process	$6.47x10^{-8}$
response to salt stress	0.000370	photosynthesis electron transport	$7.53x10^{-8}$
response to osmotic stress	0.000402	cell division	0.0000683
response to fungus	0.00143	regulation of cell size	0.001056
multidrug transport	0.00650	cell growth	0.0044
cell death	0.00675	electron transport chain	0.00716
response to oxidative stress	0.0184	M phase of cell cycle	0.049

Table 2. *Selected categories enriched in wild-type vs. gcn2 Arabidopsis seedlings after glyphosate treatment (for a complete list, see additional file 3)*

Selected categories enriched in wild-type or *gcn2* plants after an Enrichment Analysis using FatiGo (Medina et al., 2010) on the differentially expresses genes (fold-change>2 and adjusted p-values<0.05) in 16-day-old *Arabidopsis* wild-type Landsberg and *gcn2* GT8351 seedlings treated with glyphosate as described in Materials and Methods. For a complete list of GO categories (biological process) with an adjusted p-value lower than 0.05 see additional files 4 and 5).

Genotype		A_N (µmol $m^{-2}s^{-1}$)	g_s (mol $m^{-2}s^{-1}$)	C_i (µmol mol^{-1})	E (mmol $m^{-2}s^{-1}$)	PhiPS2	Fv/Fm	SPAD (a.u.)
Ler	control	7.3 a	0.13 a	301 b	3.3 a	0.106 b	0.822 NS	33 a
	Glyphosate	3.1 b	0.05 b	328 a	2.0 b	0.059	0.826	27 b
gcn2	control	7.5	0.10	288 a	3.1	0.134 NS	0.834 NS	34 b
	Glyphosate	7.2 NS	0.08 NS	255 b	2.6 NS	0.125	0.836	37 a

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

Table 3. Effect of glyphosate application on the photosynthetic rate (A_N), stomatal conductance (g_s), substomatal CO_2 concentration (C_i), transpiration rate (E), quantum efficiency of photosystem II (PhiPS2), maximum quantum yield efficiency (Fv/Fm) and SPAD index. Each value is the mean of eight independent determinations in different plants.

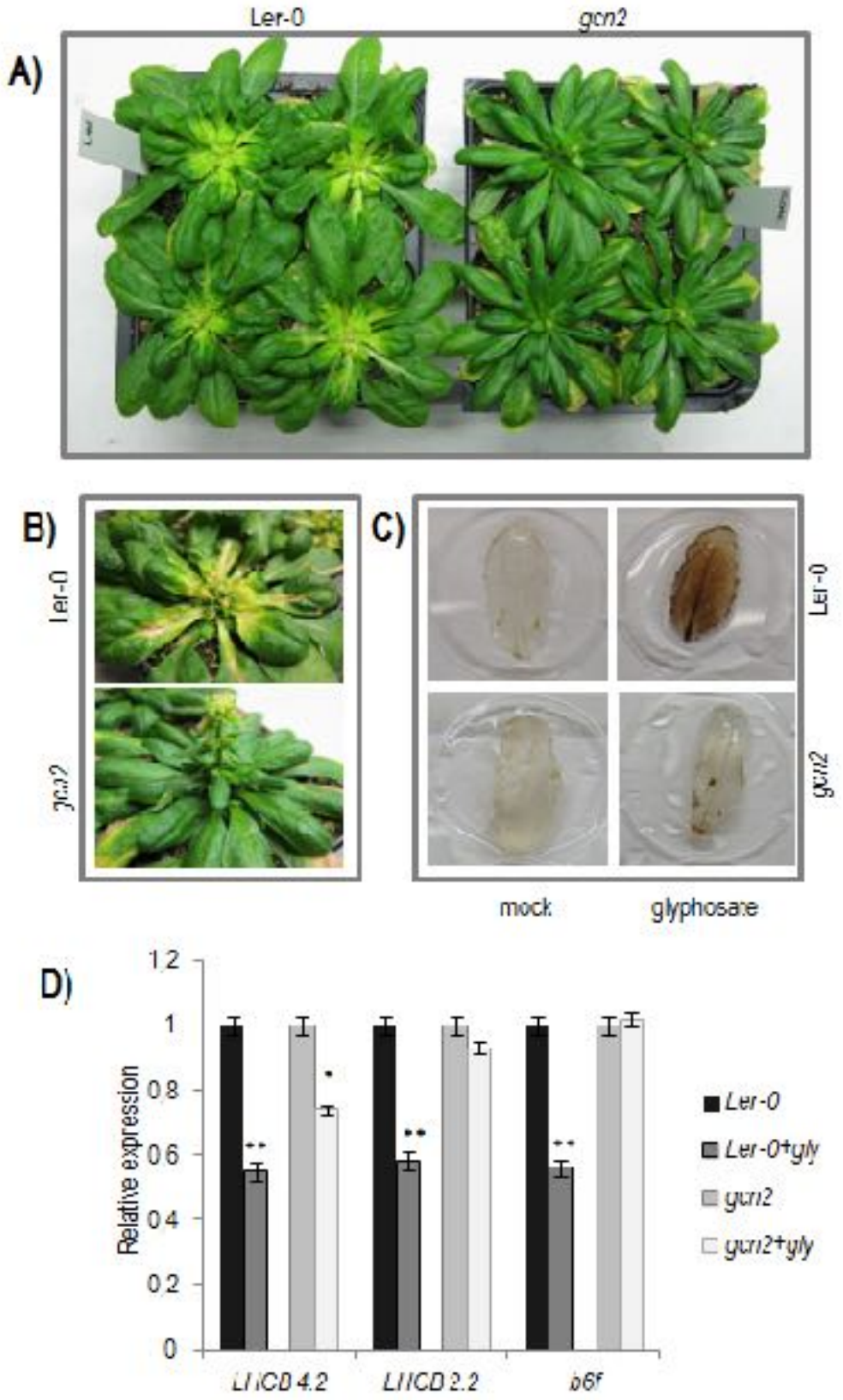


Figure 1: *Glyphosate effects over wild-type and gcn2 four-week old plants*

Pictures show aspect of four-week old *Arabidopsis* wild-type (Ler-0) and *gcn2* GT8359 plants, two weeks after glyphosate treatment. The experiment was repeated three times using 48 individual plants per genotype in every experiment. Mock-treated plants were looking similar (data not shown). General view (A) and Close-up (B) of glyphosate-treated plants. (C) Apical leaves DAB-staining of mock- and glyphosate-treated plants. Five independent plants were used as biological replicates, and two rosette leaves were sampled from each plant. The experiment was repeated three times. (D) Relative transcript levels of *LHCB 4.2* (At3g08940), *LHCB 2.2* (At2g05070), and *B6F* (At5g36120) in wild-type (Ler-0) and *gcn2* plants. Data show mean and standard error of three independent biological replicates. Each replicate contains material from five independent plants. (t-test, * P<0.05, ** P<0.01).

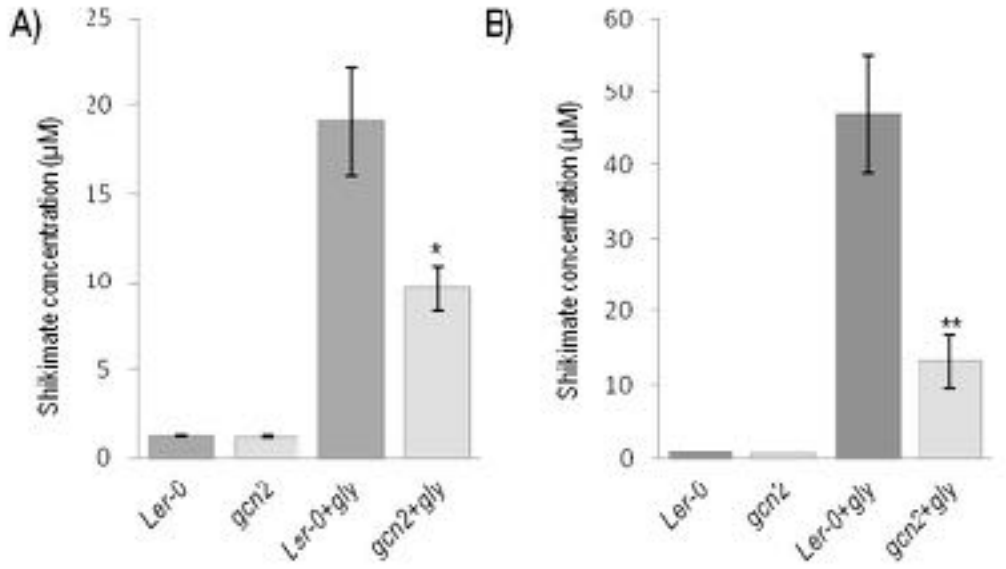
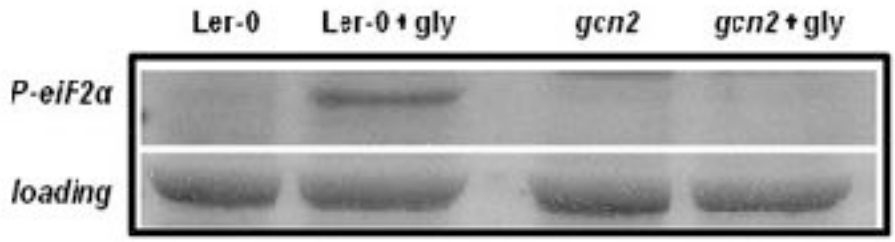


Figure 2: *gcn2 plants accumulate less shikimate than wild-type.*

Quantification of shikimate levels in seedlings (A) and adult plants (B) 72 h after mock treatment (Ler-0, *gcn2*) or glyphosate treatment (Ler-0+gly, *gcn2*+gly). Data show mean and standard error of ten independent biological replicates. Asterisks

represent significant differences between wild-type and *gcn2* plants (t-test, * $P<0.05$, ** $P<0.01$).

Additional File 1. File showing genes induced by glyphosate in 16-day-old *Arabidopsis* wild-type Landsberg and in *gcn2* mutant.



Additional File 2. File showing genes repressed by glyphosate in 16-day-old *Arabidopsis* wild-type Landsberg seedlings and in *gcn2* mutant.

Additional File 3. GO categories (biological process) enriched in 16-day-old *Arabidopsis* wild-type Landsberg seedlings treated with glyphosate compared with mock-treated plants.

Additional File 4. GO categories (biological process) enriched in 16-day-old *Arabidopsis* wild-type Landsberg seedlings treated with glyphosate compared with *gcn2*-treated plants.

Additional File 5. GO categories (biological process) enriched in 16-day-old *Arabidopsis gcn2* seedlings treated with glyphosate compared with wild-type-treated plants.

Additional Figure 1. Western blot showing inmunodetection of phosphorylated eIF2α in protein extracts of *Arabidopsis* seedlings used for microarray experiments (upper panel). Coomassie staining of a band of 45 kDa (lower panel) was used as a loading control.

3.2.ARTÍCULO 2

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

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

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Keywords: GCN1, GCN2, *Arabidopsis*, transcriptomic, eIF2α

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 At-GCN2 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* mu-

tants, 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 (54^{th} 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 de-

ficient 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 plausi-

ble 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'-GGA-CAATAATCTTGAGTCGAC-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'-GGACCTCGTC-GAGACTTTG-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'-GGA-CAATAATCTTGAGTCGAC-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 auto-fluorescence) 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.

ß-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 ß-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 ß-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

References

Alberti S, Gitler AD, Lindquist S: **A suite of Gateway cloning vectors for high-throughput genetic analysis in Saccharomyces cerevisiae**. *Yeast* 2007, **24**(10): 913-919.

Alonso R, Salavert F, Garcia-Garcia F, Carbonell-Caballero J, Bleda M, Garcia-Alonso L, Sanchis-Juan A, Perez-Gil D, Marin-Garcia P, Sanchez R, Cubuk C, Hidalgo MR, Amadoz A Hernansaiz-Ballesteros RD, Alemán A, Tarraga J, Montaner D, Medina I,

Dopazo J **Babelomics 5.0: functional interpretation for new generations of genomic data**. *Nucleic Acids Research* 2015, **43**(W1):W117-121.

Alscher RG, Erturk N, Heath LS: **Role of superoxide dismutases (SODs) in controlling oxidative stress in plants**. *Journal of Experimental Botany* 2002, **53**(372): 1331-1341.

Ajuh P, Kuster B, Panov K, Zomerdijk JC, Mann M, Lamond AI: **Functional analysis of the human CDC5L complex and identification of its components by mass spectrometry**. *Embo Journal* 2000, **19**(23):6569-6581.

Belda-Palazon B, Ruiz L, Marti E, Tarraga S, Tiburcio AF, Culianez F, Farras R, Carrasco P, Ferrando A: **Aminopropyltransferases involved in polyamine biosynthesis localize preferentially in the nucleus of plant cells**. *PLoS One* 2012, **7**(10):e46907.

Breitenbach HH, Wenig M, Wittek F, Jordá L, Maldonado-Alconada AM, Sarioglu H, Colby T, Knappe C, Bichlmeier M, Pabst E, Mackey D, Parker JE, Vlot AC **Contrasting Roles of the Apoplastic Aspartyl Protease APOPLASTIC, ENHANCED DISEASE SUSCEPTIBILITY1-DEPENDENT1 and LEGUME LECTIN-LIKE PROTEIN1 in *Arabidopsis* Systemic Acquired Resistance**. *Plant Physiology* 2014, **165**(2):791-809.

Byrne EH, Prosser I, Muttucumaru N, Curtis TY, Wingler A, Powers S, Halford NG: **Overexpression of GCN2-type protein kinase in wheat has profound effects on free amino acid concentration and gene expression**. *Plant Biotechnology Journal* 2012, **10**(3):328-340.

Castilho BA, Shanmugam R, Silva RC, Ramesh R, Himme BM, Sattlegger E: **Keeping the eIF2 alpha kinase Gcn2 in check**. *Biochimica et Biophysica Acta* 2014, **1843**(9):1948-1968.

Curtis MD, Grossniklaus U. **A gateway cloning vector set for high-throughput functional analysis of genes in planta**. *Plant Physiology*. 2003 Oct;133(2):462-9

Faus I, Zabalza A, Santiago J, Nebauer SG, Royuela M, Serrano R, Gadea J: **Protein kinase GCN2 mediates responses to glyphosate in *Arabidopsis***. *BMC Plant Biology* 2015, **15**:14.

Fernandez AP, Strand A: **Retrograde signaling and plant stress: plastid signals initiate cellular stress responses**. *Current Opinion in Plant Biology* 2008, **11**(5): 509-513

Feys BJ, Wiermer M, Bhat RA, Moisan LJ, Medina-Escobar N, Neu C, Cabral A, Parker JE: ***Arabidopsis* SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity**. *The Plant Cell* 2005, **17**(9):2601-2613.

Flexas J, Ortuno MF, Ribas-Carbo M, Diaz-Espejo A, Florez-Sarasa ID, Medrano H: **Mesophyll conductance to CO2 in *Arabidopsis thaliana***. *New Phytology* 2007, **175**(3):501-511.

Flores-Perez U, Jarvis P: **Molecular chaperone involvement in chloroplast protein import**. *Biochimica et Biophysica Acta* 2013, **1833**(2):332-340.

Garcia-Barrio M, Dong JS, Ufano S, Hinnebusch AG: **Association of GCN1-GCN20 regulatory complex with the N-terminus of eIF2 alpha kinase GCN2 is required for GCN2 activation**. *Embo Journal* 2000, **19**(8):1887-1899.

Genty B, Briantais JM, Baker NR: **The Relationship between the Quantum Yield of Photosynthetic Electron-Transport and Quenching of Chlorophyll Fluorescence**. *Biochimica et Biophysica Acta* 1989, **990**(1):87-92.

Hinnebusch AG: **Translational regulation of GCN4 and the general amino acid control of yeast**. *Annual Review in Microbiology* 2005, **59**:407-450.

Immanuel TM, Greenwood D, MacDiarmid RM: **A critical review of translation initiation factor eIF2α kinases in plants-regulating protein synthesis during stress.** *Functional Plant Biology* 2012, **39**:717-735.

Jarvis P, Chen LJ, Li H, Peto CA, Fankhauser C, Chory J: **An *Arabidopsis* mutant defective in the plastid general protein import apparatus**. *Science* 1998, **282**(5386):100-103.

Johnston AJ, Meier P, Gheyselinck J, Wuest SEJ, Federer M, Schlagenhauf E, Becker JD, Grossniklaus U: **Genetic subtraction profiling identifies genes essential for *Arabidopsis* reproduction and reveals interaction between the female gametophyte and the maternal sporophyte**. *Genome Biology* 2007, **8**(10).

Kwon KC, Cho MH: **Deletion of the chloroplast-localized AtTerC gene product in *Arabidopsis thaliana* leads to loss of the thylakoid membrane and to seedling lethality**. *Plant Journal* 2008, **55**(3):428-442.

Li MW, AuYeung WK, Lam HM **The GCN2 homologue in *Arabidopsis thaliana* interacts with uncharged tRNA and uses *Arabidopsis* eIF2α molecules as direct substrates.** *Plant Biology (Stuttgart)* 2013, **15**(1):13-18.

Lintala M, Allahverdiyeva Y, Kidron H, Piippo M, Battchikova N, Suorsa M, Rintamaki E, Salminen TA, Aro E-M, Mulo P: **Structural and functional characterization of ferredoxin-NADP(+)-oxidoreductase using knock-out mutants of *Arabidopsis***. *Plant Journal* 2007, **49**(6):1041-1052.

Lintala M, Lehtimaki N, Benz JP, Jungfer A, Soll J, Aro EM, Bolter B, Mulo P: **Depletion of leaf-type ferredoxin-NADP(+) oxidoreductase results in the permanent induction of photoprotective mechanisms in *Arabidopsis* chloroplasts**. *Plant Journal* 2012, **70**(5):809-817.

Marton M.J., Crouch D. & Hinnebusch A.G. **GCN1, a translational activator of GCN4 in Saccharomyces cerevisiae, is required for phosphorylation of eukaryotic translation initiation factor 2 by protein kinase GCN2**. *Molecular and Cellular Biology*. 1993. Jun;13(6):3541-56.

Marton MJ, deAldana CRV, Qiu HF, Chakraburtty K, Hinnebusch AG: **Evidence that GCN1 and GCN20, translational regulators of GCN4, function on elongating ribosomes in activation of eIF2 alpha kinase GCN2**. *Molecular and Cellular Biology* 1997, **17**(8):4474-4489.

Miura E, Kato Y, Sakamoto W: **Comparative transcriptome analysis of green/white variegated sectors in *Arabidopsis* yellow variegated2: responses to oxidative and other stresses in white sectors**. *Journal of Experimental Botany* 2010, **61**(9): 2433-2445.

Monaghan J, Li X: **The HEAT Repeat Protein ILITYHIA is Required for Plant Immunity**. *Plant and Cell Physiology* 2010, **51**(5):742-753.

Mur L.A. KP, Lloyd A.J., Ougham H. Prats, E: **The hypersensitive response; the centenary is upon us but how much do we know?** . *Journal of Experimental Botany* 2008, **59**:501-520.

Nameki N, Yoneyama M, Koshiba S, Tochio N, Inoue M, Seki E, Matsuda T, Tomo Y, Harada T, Saito K, Kobayashi N, Yabuki T, Aoki M, Nunokawa E, Matsuda N, Sakagami N, Terada T Shirouzu M, Yoshida M, Hirota H, Osanai T, Tanaka A, Arakawa T, Carninci P, Kawai J, Hayashizaki Y, Kinoshita K, Güntert P, Kigawa T, Yokoyama S. **Solution structure of the RWD domain of the mouse GCN2 protein**. *Protein Science*. 2004. Aug;13(8):2089-100.

Nukazuka A, Fujisawa H, Inada T, Oda Y, Takagi S: **Semaphorin controls epidermal morphogenesis by stimulating mRNA translation via eIF2 alpha in Caenorhabditis elegans**. *Genes & Development* 2008, **22**(8):1025-1036.

op den Camp RG, Przybyla D, Ochsenbein C, Laloi C, Kim C, Danon A, Wagner D, Hideg E, Göbel C, Feussner I, Nater M, Apel K **Rapid induction of distinct stress responses after the release of singlet oxygen in *arabidopsis***. *The Plant Cell* 2003, **15**(10):2320-2332.

Paddock TN, Mason ME, Lima DF, Armstrong GA: ***Arabidopsis* protochlorophyllide oxidoreductase A (PORA) restores bulk chlorophyll synthesis and normal development to a porB porC double mutant**. *Plant Molecular Biology* 2010, **72**(4-5): 445-457.

Pereira CM, Sattlegger E, Jiang HY, Longo BM, Jaqueta CB, Hinnebusch AG, Wek RC, Mello LE, Castilho BA. **IMPACT, a protein preferentially expressed in the mouse brain, binds GCN1 and inhibits GCN2 activation**. *Journal of Biological Chemistry*. Aug .2005, 5;280(31):28316-23.

Rajan VBV, D'Silva P: ***Arabidopsis thaliana* J-class heat shock proteins: cellular stress sensors**. *Functional & Integrative Genomics* 2009, **9**(4):433-446.

Rojas M. Gingras AC, Dever TE. **Protein phosphatase PP1/GLC7 interaction domain in yeast eIF2γ bypasses targeting subunit requirement for eIF2α dephosphorylation.** . *Proceedings of the Natural Academy of Science U S A* 2014, **111**(14): 1344-1353.

Roy B, von Arnim AG: **Translational Regulation of Cytoplasmic mRNAs**. *Arabidopsis Book* 2013, **11**:e0165.

Sanchez-Fernandez R, Davies TG, Coleman JO, Rea PA: **The *Arabidopsis thaliana* ABC protein superfamily, a complete inventory**. *Journal of Biological Chemistry* 2001, **276**(32):30231-30244.

Sattlegger E, Hinnebusch AG: **Separate domains in GCN1 for binding protein kinase GCN2 and ribosomes are required for GCN2 activation in amino acid-starved cells**. *Embo Journal* 2000, **19**(23):6622-6633.

Supek F, Bosnjak M, Skunca N, Smuc T: **REVIGO summarizes and visualizes long lists of gene ontology terms**. *PLoS One* 2011, **6**(7):e21800.

Tusher VG, Tibshirani R, Chu G: **Significance analysis of microarrays applied to the ionizing radiation response**. *Proceedings of the Natural Academy of Science U S A* 2001, **98**(9):5116-5121.

Wang L, Li H, Zhao C, Li S, Kong L, Wu W, Kong W, Liu Y, Wei Y, Zhu JK, Zhang H **The inhibition of protein translation mediated by AtGCN1 is essential for cold tolerance in *Arabidopsis thaliana***. *Plant Cell and Environment* 2016, **40**(1):56-68.

Wang Q, Sullivan RW, Kight A, Henry RL, Huang J, Jones AM, Korth KL: **Deletion of the chloroplast-localized Thylakoid formation1 gene product in *Arabidopsis* leads to deficient thylakoid formation and variegated leaves**. *Plant Physiology* 2004, **136**(3):3594-3604.

Wildermuth MC, Dewdney J, Wu G, Ausubel FM: **Isochorismate synthase is required to synthesize salicylic acid for plant defence**. *Nature* 2001, **414**(6863): 562-565.

Yang Y, Zhang Y, Ding P, Johnson K, Li X, Zhang Y: **The ankyrin-repeat transmembrane protein BDA1 functions downstream of the receptor-like protein SNC2 to regulate plant immunity**. *Plant Physiology* 2012, **159**(4):1857-1865.

Yang R, Wek SA, Wek RC: **Glucose limitation induces GCN4 translation by activation of Gcn2 protein kinase**. *Molecular and Cellular Biology* 2000, **20**(8):2706-2717

Yeh Y-H, Chang Y-H, Huang P-Y, Huang J-B, Zimmerli L: **Enhanced *Arabidopsis* pattern-triggered immunity by overexpression of cysteine-rich receptor-like kinases**. *Frontiers in Plant Science* 2015, **6**.

Zeng W, Brutus A, Kremer JM, Withers JC, Gao X, Jones AD, He SY: **A Genetic Screen Reveals *Arabidopsis* Stomatal and/or Apoplastic Defenses against Pseudomonas syringae pv. tomato DC3000**. *Plos Pathogens* 2011, **7**(10).

Zhang Y, Dickinson JR, Paul MJ, Halford NG: **Molecular cloning of an *arabidopsis* homologue of GCN2, a protein kinase involved in co-ordinated response to amino acid starvation**. *Planta* 2003, **217**(4):668-675.

Zhang Y., Wang Y., Kanyuka K., Parry M.A., Powers S.J., & Halford N.G. **GCN2-dependent phosphorylation of eukaryotic translation initiation factor-2alpha in *Arabidopsis***. 2008 *Journal of Experimental Botany*. 59(11):3131-41.

Zurbriggen M.D., Carrillo N., Tognetti V.B., Melzer M., Peisker M., Hause B. Hajirezaei M.R. **Chloroplast-generated reactive oxygen species play a major role in localized cell death during the non-host interaction between tobacco and Xanthomonas campestris pv. vesicatoria**. *Plant Journal*. 2009 Dec; 60 (6):962-73.

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	0.2 b	321	3.4 b	0.2	52	2847	0.814	22
	ila3	9.5	4 a	332	4.5 a	28	6	2627	0.809	21
	gcn2-2	9.6 NS	0.3 b	323 NS	3.5 b	0.2 NS	49 NS	2829 NS	0.817 NS	24 NS
			3			22	1			
			0.2			0.2	51			
			5			19	4			
Young	Col-0	8.1 a	0.1	290 a	2.3	0.2 a	55 a	2916 a	0.811 a	22 a
	ila3	4.8 b	8	342 b	2.7	10 b	0 b	2328 b	0.794 b	15 b
	gcn2-2	7.8 c	0.2 NS	316 a	2.8 NS	0.1 a	46 a	3007 a	0.825 a	23 a
			0			85	0			
			0.2			0.2	52			
			0			11	4			

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

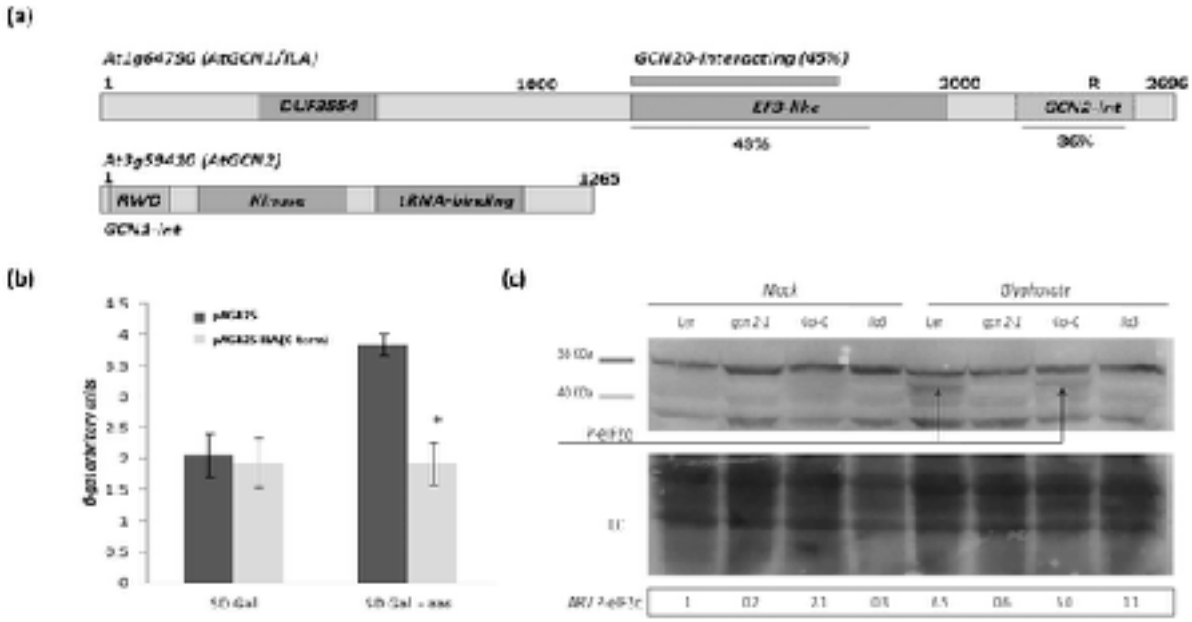


Figure 1. Interaction of ILA with GCN2. (a) Protein structure of At1g64790 ILITHYIA (ILA) and At3g59410 (AtGCN2), showing conserved domains of both proteins. Amino acid positions are indicated. Identity (BLASP) with the corresponding yeast (*Saccharomyces cerevisiae*) GCN1 protein is shown in percentage 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)** ß-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.

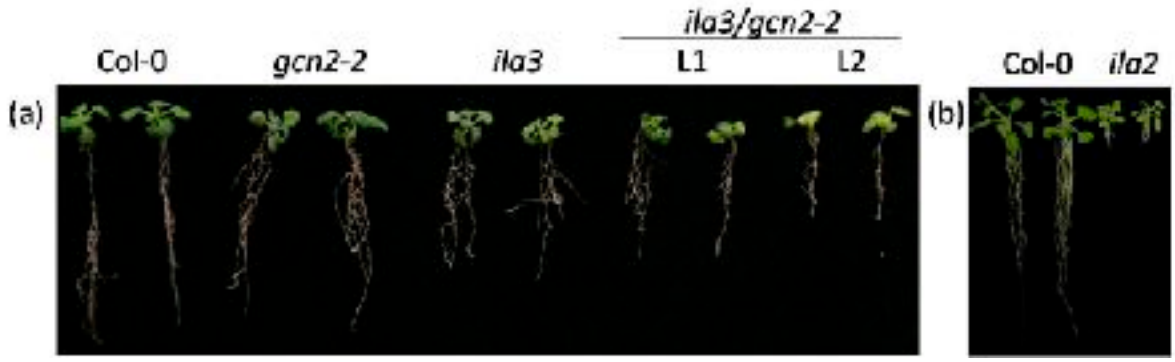


Figure 2. Root development is impaired in *ila* mutants. Representative roots of Col-0, *gcn2-2, ila3*, and two different lines of *ila3/gcn2-2* double mutant **(a)** as well as *ila2* **(b)** after growing vertically on MS medium during 20 days.

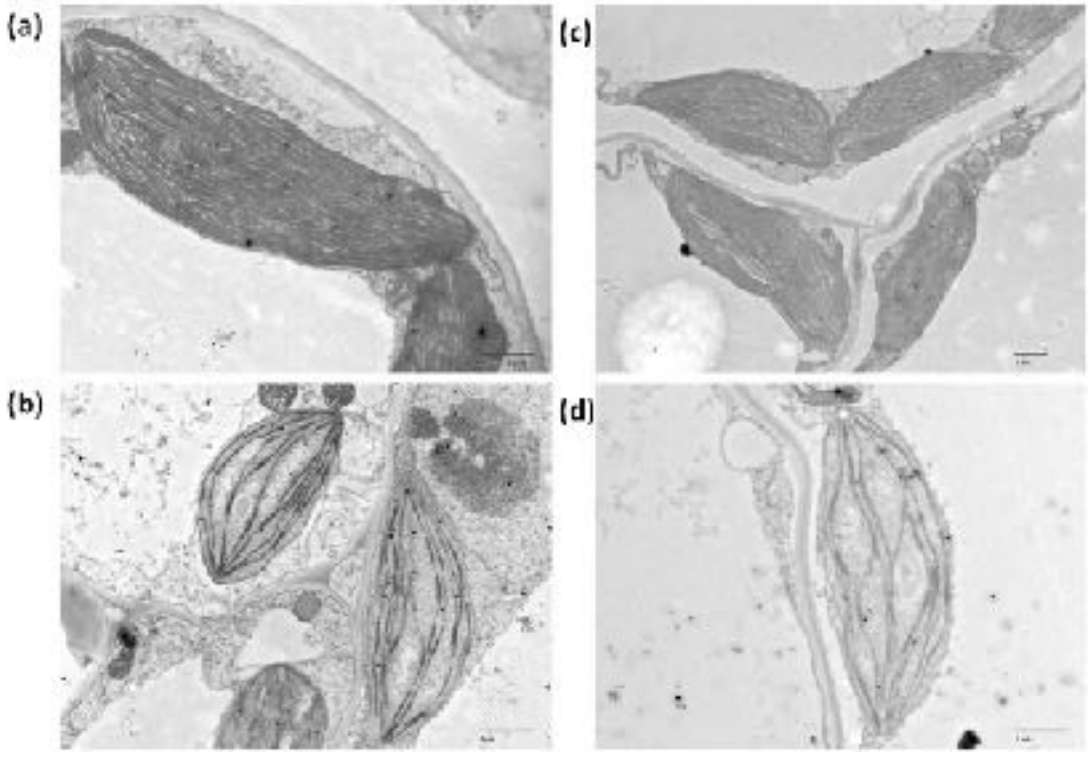


Figure 3. *Ila* mutants have defective chloroplast development. Transmission electron microscopy images of Col-0 **(a)**, *ila3* **(b)**, *gcn2-2* **(c)** and *ila2* **(d)** leaves chloroplasts showing internal thylakoid membranes. Plants were grown for 30 days in the greenhouse under long-day conditions.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

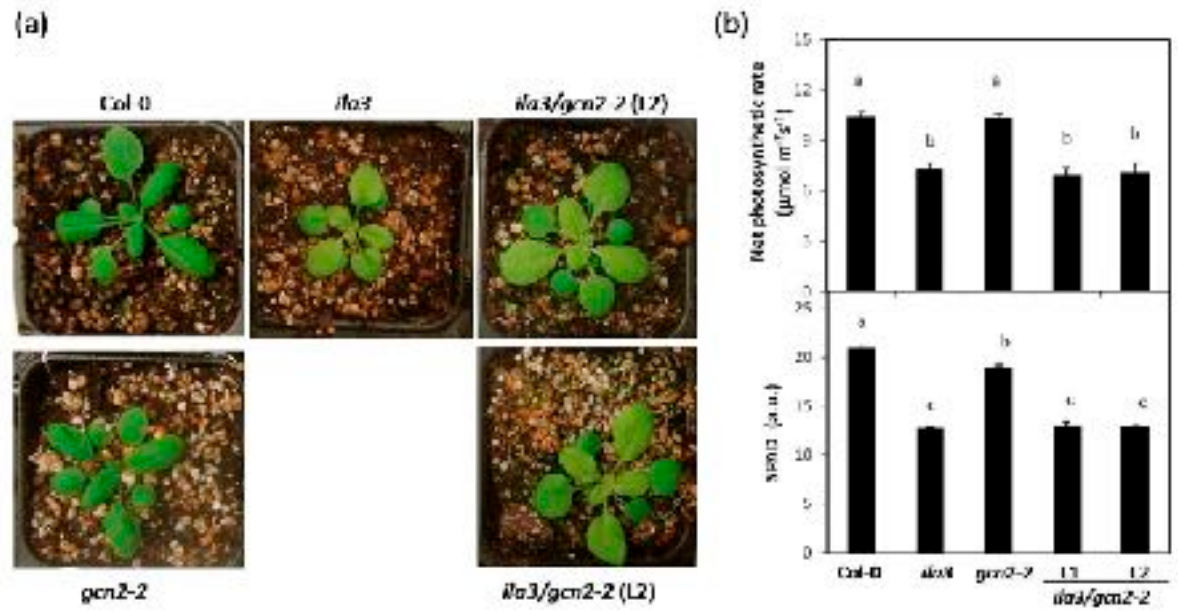


Figure 4. *ila3/gcn2-2* double mutants maintain the chlorotic phenotype characteristic of the *ila3* mutant. (a) Rosette phenotype of plants grown in the greenhouse under long-day conditions for 20 days. **(b)** Histograms showing the photosynthetic rate (AN) and SPAD index in wild-type (Col-0), *gcn1*, *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.

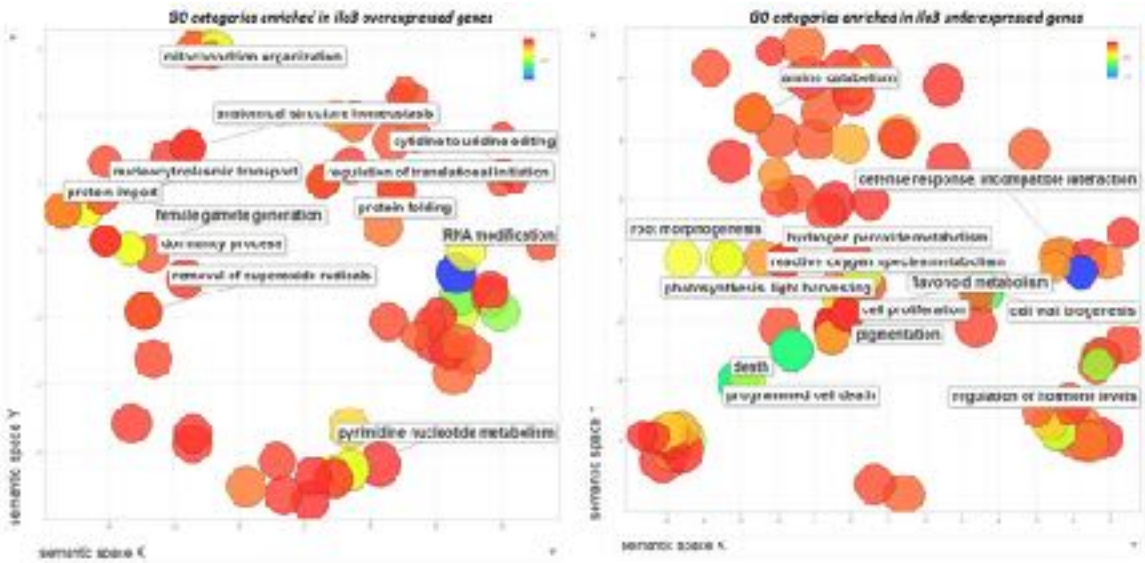
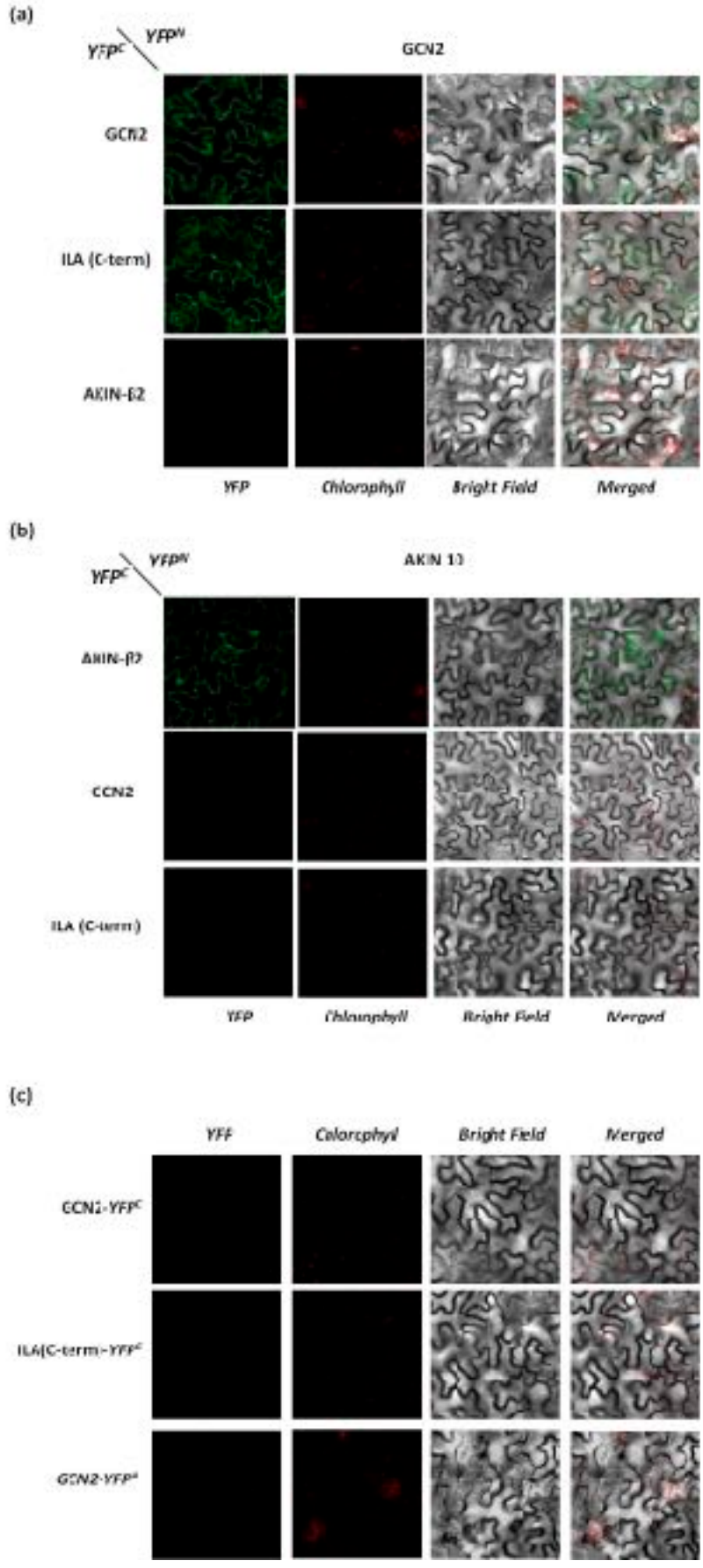
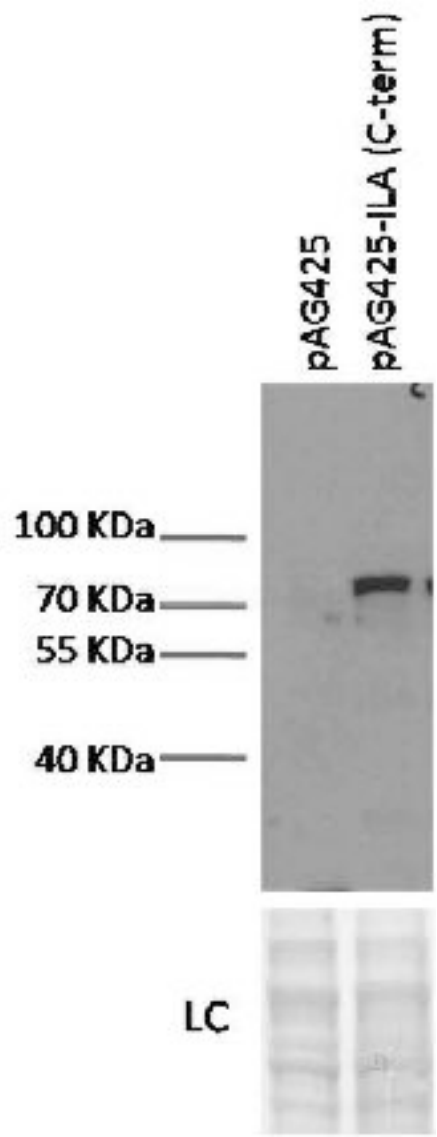


Figure 5. Representative GO categories enriched in *ila3* overexpressed and underexpressed genes. The scatterplot shows the cluster representatives (terms remaining after the redundancy reduction) in a two dimensional space derived by applying multidimensional scaling to a matrix of the GO terms' semantic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

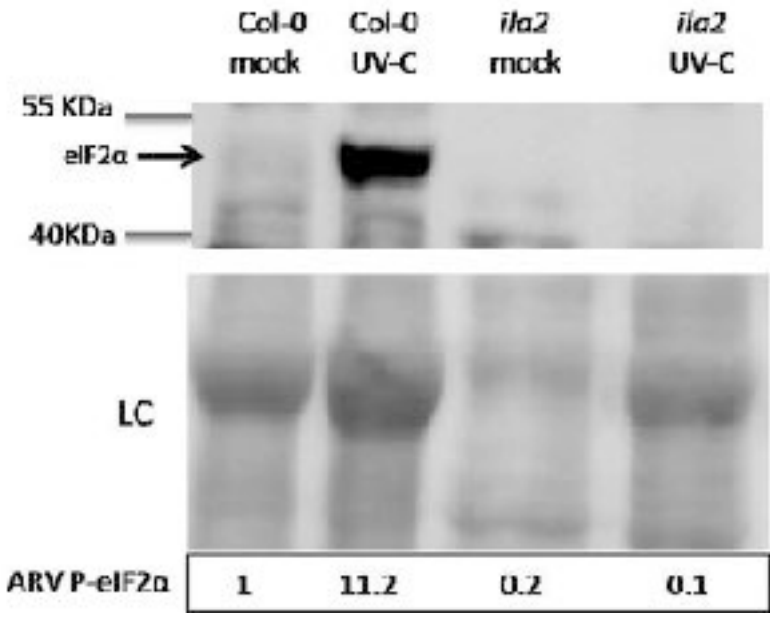


Supplemental Figure 1: Bimolecular fluorescence complementation (BiFC) assay between AtGCN2 and the proteins indicated. Images were obtained from the YFP

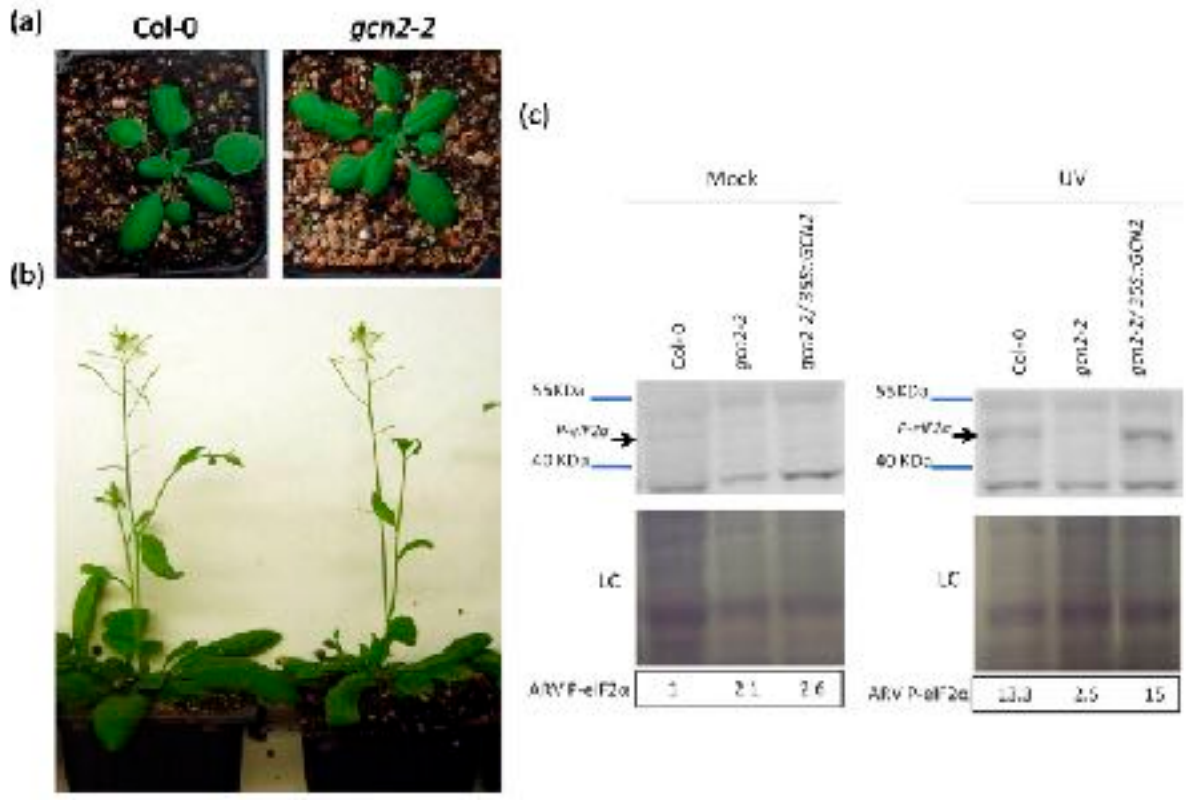
channel, chlorophyll channel, bright field and merged panel (see Materials and Methods). The following negative controls are included (AKINβ2- YFP^C /GCN2- YFP^N; GCN2- YFP^C / AKIN10- YFP^N; ILA-C-term- YFP^C /AKIN10- YFP^N. The following positive control is included: AKINβ2- YFP^C/ AKIN10- YFP^N. Autofluorescence is discarded for GCN2- YFP^C, ILA-C-term- YFP^C and GCN2- YFP^N.



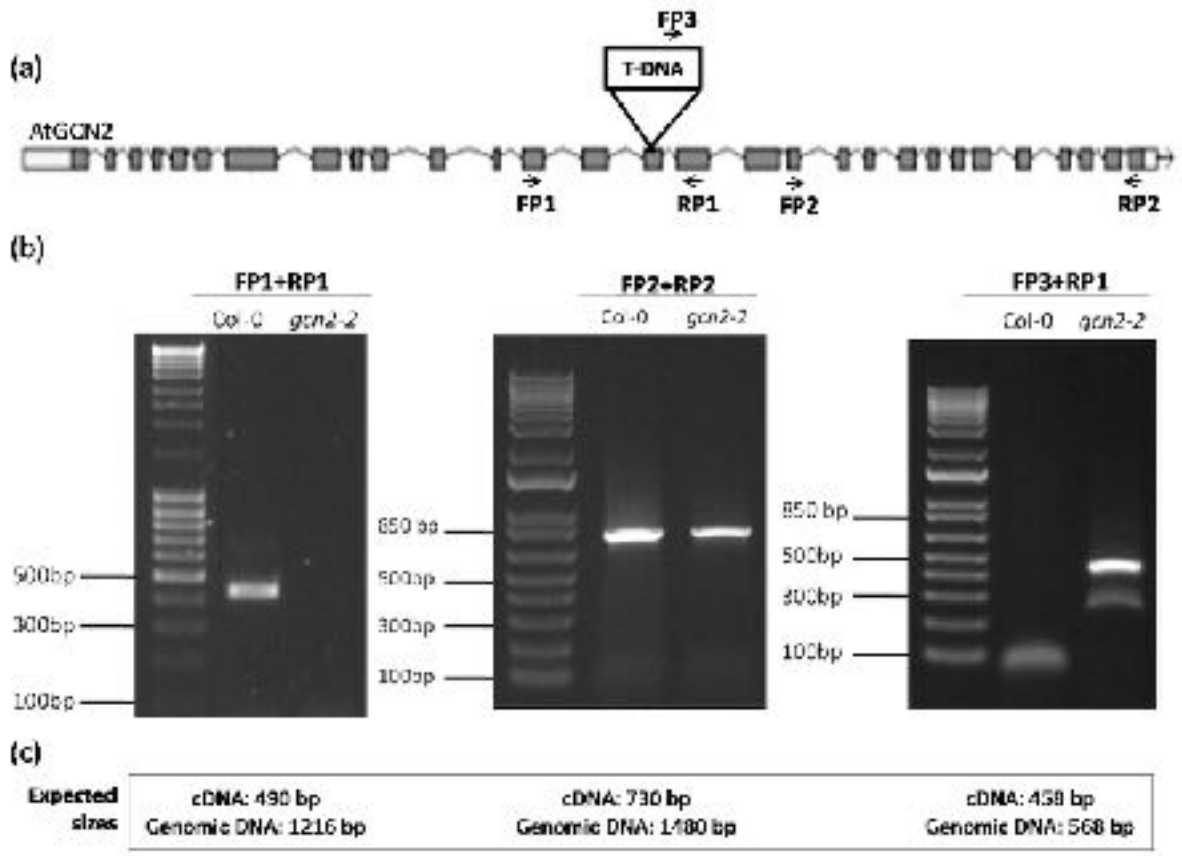
Supplemental Figure 2. Western blot (HA antibody) showing the expression of ILA(C-term) in W303 yeast cells harboring the p180 plasmid transformed with the pAG425 empty vector or with pAG425-ILA(C-term). A band of approximately 70 kDa is observed, corresponding to the expected size of the ILA(C-term)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

3.3.ARTÍCULO 3

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SHORT COMMUNICATION

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

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

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Running head: differentially-expressed proteins in the *ila2* mutant in *Arabidopsis*

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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 eIF2α 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. In order to gain insight into this new function of the ILA protein, we have performed proteomic analysis to identify proteins differentially-expressed in the strong loss-of-function alleles 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 AG, 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 exist in all plant species studied so far, and eIF2α is phosphorylated by many stress 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-eIF2α

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-eIF2α regulation in plants under stress.

A key component of this signalling pathway is the protein GCN1, necessary in yeast and animals for eIF2α phosphorylation. GCN1 promotes GCN2 activation by 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 eIF2α (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 is 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 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).

In our 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 seedlings 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% ß-mercaptoethanol.and centrifuged for 20min. 1% TCA was added to supernatant at 1:1 v/v. and incubated for 1 h. Samples were centrifuged for 20m and washed three time with cold acetone.

Equal amounts (50 µg) 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 µL 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 µL of IEF rehydration buffer, containing the reagents Destreak and Pharmalyte, pH 3–10. The CyDyes-labelled pool sample (150 µg) 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 for3 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, distained 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,pH7.8; 5 mM $CaCl_2$; 12.5 ng/µL) (o/n at 37 °C).

(The digestion was stopped with 7 mL of 0.1% 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 a 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 µL/min, and peptides were trapped onto a PepMap C18 pre-column (5mm×300mm i.d.). Peptides were then eluted onto the PepMap C18 analytical column (15 cm×75mm 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 reveal 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 *ila 2* seedlings will determine, first, to which extend 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 *ila2* seedlings, as well as a composite image of both, is shown in Figure 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 were excised from 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 in 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 Figure1B.

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, the weak *ila3* and the strong *ila2* alleles' chloroplasts 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 CO_2 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 these defects 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 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 preinitiation complex. Similar to *ila2*, those plants present a reduced expression of CA1, RCA and PSBQ. In summary, the link between chloroplast missfunction and defective plant development is strengthen 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 Figure 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 eiF2α kinase GCN2, which upon phosphorylation of eiF2α, 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 eiF2α 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 translation of GCN4 in yeast (Singh et al., 2006) and ATF4 in *Drosophila* (Kozel et al., 2016). For the first time, this evidence 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 cause 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 cause 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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References

Carmona L, Alquézar B, Tárraga S, Peña L. (2019). Protein analysis of moro blood orange pulp during storage at low temperatures. Food Chem. Mar 30;277:75-83.

Chen Z, Jolley B, Caldwell C, Gallie DR. (2014) Eukaryotic translation initiation factor eIFiso4G is required to regulate violaxanthin De-epoxidase expression in *Arabidopsis*. J Biol Chem. May 16; 289(20):13926-36.

DiMario RJ, Clayton H, Mukherjee A, Ludwig M, Moroney JV. (2017). Plant Carbonic Anhydrases: Structures, Locations, Evolution, and Physiological Roles. Mol Plant. Jan 9; 10(1): 30-46.

Faus I, Zabalza A, Santiago J, Nebauer SG, Royuela M, Serrano R, Gadea J. (2015). Protein kinase GCN2 mediates responses to glyphosate in *Arabidopsis*. BMC Plant Biology, 15:14.

Faus I, Niñoles R, Kesari V, Llabata P, Tam E, Nebauer SG, Santiago J, Hauser MT, Gadea J. (2018). *Arabidopsis* ILITHYIA protein is necessary for proper chloroplast biogenesis and root development independent of eIF2α phosphorylation. J Plant Physiol. May - Jun;224-225:173-182.

Fromm S, Braun HP, Peterhansel C. (2016) Mitochondrial gamma carbonic anhydrases are required for complex I assembly and plant reproductive development. New Phytol. Jul; 211(1):194-207.

Garcia-Barrio, Minerva & Dong, Jinsheng & Ufano, Sandra & Hinnebusch, Alan. (2000). Association of GCN1-GCN20 regulatory complex with the N-terminus of eIF2α kinase GCN2 is required for GCN2 activation. The EMBO Journal. 19. 1887-1899. 10.1093/emboj/19.8.1887.

Hinnebusch AG (2005). Translational regulation of GCN4 and the general amino acid control of yeast. Annual Review in Microbiology, 59:407-450.

Izquierdo Y, Kulasekaran S, Benito P, López B, Marcos R, Cascón T, Hamberg M, Castresana C. (2018) *Arabidopsis* nonresponding to oxylipins locus NOXY7 encodes a yeast GCN1 homolog that mediates noncanonical translation regulation and stress adaptation. Plant Cell Environ. Jun;41(6):1438-1452.

Kozel C, Thompson B, Hustak S, et al. Overexpression of eIF5 or its protein mimic 5MP perturbs eIF2 function and induces ATF4 translation through delayed re-initiation. Nucleic Acids Res. 2016;44(18):8704–8713. doi:10.1093/nar/gkw559

Krishna P, Gloor G. (2001) The Hsp90 family of proteins in *Arabidopsis thaliana*. Cell Stress Chaperones.;6(3):238–246.

Lellis AD, Allen ML, Aertker AW, Tran JK, Hillis DM, Harbin CR, Caldwell C, Gallie DR, Browning KS. (2010) Deletion of the eIFiso4G subunit of the *Arabidopsis* eIFiso4F translation initiation complex impairs health and viability. Plant Mol Biol. Oct;74 (3):249-63.

Lintala M, Lehtimäki N, Benz JP, Jungfer A, Soll J, Aro EM, Bölter B, Mulo P (2012). Depletion of leaf-type ferredoxin-NADP(+) oxidoreductase results in the permanent induction of photoprotective mechanisms in *Arabidopsis* chloroplasts. Plant J. Jun;70(5): 809-17.

Mollapour M, Neckers L. (2012) Post-translational modifications of Hsp90 and their contributions to chaperone regulation. Biochim Biophys Acta.;1823(3):648–655. doi:10.1016/j.bbamcr.2011.07.018

Monaghan J, Li X. (2010) The HEAT repeat protein ILITYHIA is required for plant immunity. Plant Cell Physiol. May;51(5):742-53.

Portis AR Jr, Li C, Wang D, Salvucci ME. (2008) Regulation of Rubisco activase and its interaction with Rubisco. J Exp Bot.;59(7):1597-604.

Sharma DK, Bressler K, Patel H, Balasingam N, Thakor N. (2016) Role of Eukaryotic Initiation Factors during Cellular Stress and Cancer Progression. J Nucleic Acids.; 2016:8235121.

Singh C.R., Lee B., Udagawa T., Mohammad-Qureshi S.S., Yamamoto Y., Pavitt G.D., Asano K. An eIF5/eIF2 complex antagonizes guanine nucleotide exchange by eIF2B during translation initiation. EMBO J. 2006;25:4537–4546.

Wang L, Li H, Zhao C, Li S, Kong L, Wu W, Kong W, Liu Y, Wei Y, Zhu JK, Zhang H. (2017) The inhibition of protein translation mediated by AtGCN1 is essential for cold tolerance in *Arabidopsis thaliana*. Plant Cell Environ. Jan;40(1):56-68.

Zhang Y., Wang Y., Kanyuka K., Parry M.A., Powers S.J., & Halford N.G. (2008) GCN2-dependent phosphorylation of eukaryotic translation initiation factor-2alpha in *Arabidopsis*. Journal of Experimental Botany. 59(11):3131-41.

Zhou X, Liao WJ, Liao JM, Liao P, Lu H. (2015) Ribosomal proteins: functions beyond the ribosome. J Mol Cell Biol.;7(2):92–104. doi:10.1093/jmcb/mjv014

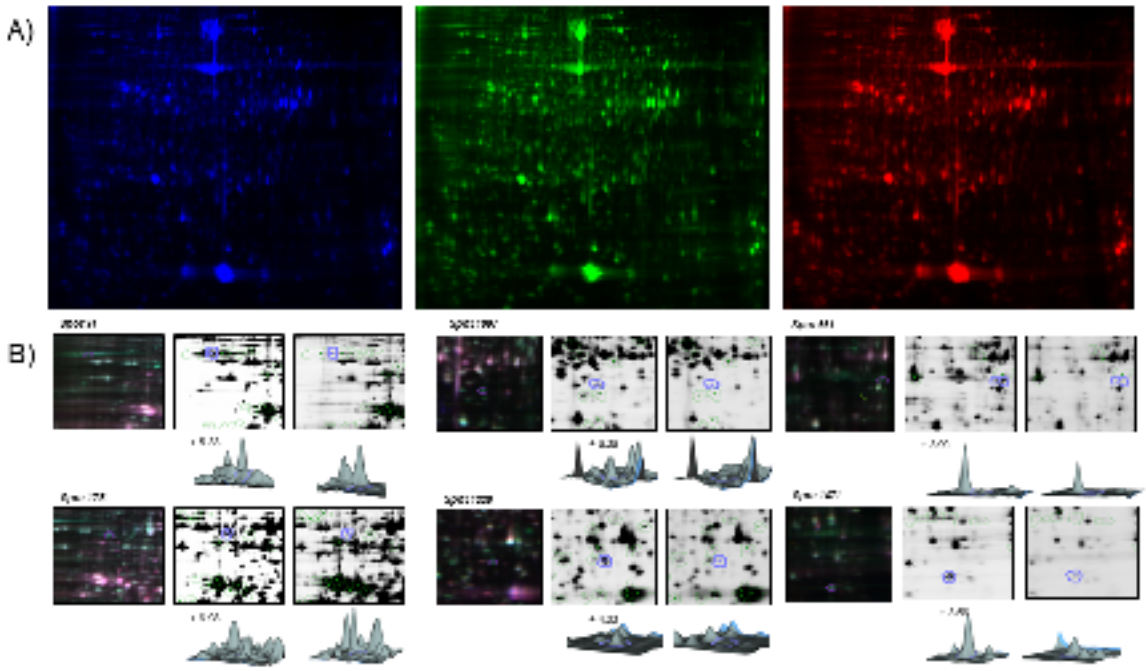

Figure 1.

2D-DIGE fluorescent labelled proteins. A) Representative side by side comparison of gel images showing the differently-labelled proteins from wild type (Cy5) and the irradiated sample (Cy3), as well as pooled sample user for comaprison (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. C) Volumetric quantification of representative spots of interest identified. Abundance values are expressed as the ratio obtained from volumetric image comparisons.

Table 1

List of differentially accumulated proteins identified between wild type and *ila2* seedlings.

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

Spot no.	Ratio ila2/wt	Acc. num	Protein pilot score	AGI name	Gene name	Prot. annotation	Functional classification
175	6.98	sp|Q8GUM7|HSP71_ ARATH	119.49	At4g37910	HSP70-4	Heat shock protein	Molecular chaperone
1097	6.36	sp|Q9FIV9|R1212_ ARATH	4.01	At1g57660	RPL21E	60S ribosomal protein	Ribosomal protein
91	5.33	sp|P51818|HS90T3_ ARATH	39.68	At5g56010	HSP90-3	Heat shock protein	Molecular chaperone
1778	4.03	sp|Q8L7S0|RBG7_ ARATH	26.22	At2g21660	GRP7	Glycine-rich RNA-binding protein	RNA transcription or processing during stress
661	3.99	sp|P49227|RL52_ ARATH	53.33	At5g39740	RPL5B	60S ribosomal protein	Ribosomal protein
1471	3.56	sp|Q8RXU5|R37A2_ ARATH	7.83	At3g60245	RPL37AC	60S ribosomal protein	Ribosomal protein
873	3.54	sp|Q8LH56|RAN3_ ARATH	31.12	At5g55190	RAN3	GTP-binding nuclear protein	Nucleocytoplasmic transport
1308	3.08	sp|Q93VR4|ML423_ ARATH	27.13	At1g24020	MLP423	MLP-like protein	Abscisic acid signalling pathway
1174	2.67	sp|Q93VP3|IF5A2_ ARATH	15.96	At1g26630	EIF5A-2	Eukaryotic translation initiation factor 5A-2	Protein biosynthesis
243	2.21	sp|Q8L7B5|CH60B_ ARATH	131.94	At2g33210	HSP60-2	Heat shock protein	Molecular chaperone
435	2.13	sp|Q9LUT2|METK4_ ARATH	59.85	At3g17390	METK4	S-Adenosylmethionine synthase 4	Lignin biosynthetic process
664	− 5.12	sp|Q8W093|FNRL2_ ARATH	37.48	At1g20020	FNR2	Ferredoxin NADP reductase	Photosynthesis
927	− 3.43	sp|P27140|BCA1_ ARATH	33.18	At3g01500	BCA1	Beta carbonic anhydrase	Photosynthesis
1289	− 3.21	sp|Q41932|PSBQ2_ ARATH	52.82	At1g05180	PBSQ2	Photosystem II subunit	Photosystem II assembly
702	− 3.16	sp|Q96262|PCAP1_ ARATH	12.92	At1g20260	PCAP1	Plasma membrane associated cation-binding protein	Response to abiotic stress
498	− 3.15	sp|P25697|KPPR_ ARATH	60.83	At1g32060	PRK	Phosphoribulokinase	Calvin cycle, photosynthesis
882	− 2.24	sp|P10795|RBS1A_ ARATH	26.01	At1g67090	RBCS-1A	Ribulose bisphosphate carboxylase small chain	Calvin cycle, photosynthesis
627	− 2.22	sp|P10896|RCA_ ARATH	55.12	At2g39730	RCA	RuBisCO activase	Activation of RuBisCO

Supplemental Table 1.

Complete Lists of differential spots between wild type and *ila2* samples, showing the number of gels were the spot appeared, the FDR corrected p-value after the t-test, and the average fold-ratio *ila2*/wild type.

3.4.ARTÍCULO 4

Artículo en revisión en la revista: Plant Molecular Biology Reporter

GCN20 loss-of-function mutant analysis in *Arabidopsis* reinforces a link with GCN1 but not with GCN2 in stress and development.

GCN20 loss-of-function mutant analysis in *Arabidopsis* reinforces a link with GCN1 but not with GCN2 in stress and development.

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Key Message

Transcriptomic experiments and correlation of phenotypes under abiotic stress of *gcn1* and *gcn20* plants reinforce a link between these two proteins, independent of the conserved kinase GCN2.

Abstract

One of the main mechanisms regulating translation is the one based on the phosphorylation of the eIF2α initiation factor by the GCN2 protein kinase. In yeast, GCN2 binds to the GCN1 and GCN20 proteins, facilitating the activation of the kinase on translating ribosomes. Homologous of the three proteins exist in *Arabidopsis*. In this species, whereas the GCN2 kinase is activated under several stress situations, the involvement of GCN1 and GCN20 in these processes is controversial, and a new role for GCN1 in translation, independent of GCN2, has been proposed. We show here that any of the five GCN20-like genes in *Arabidopsis* is needed for eIF2α phosphorylation. Furthermore, plant phenotypes under abiotic stresses and chloroplast development suggest that GCN20 is functionally linked with GCN1, but not with GCN2. Finally, *gcn1* and *gcn20* mutants share similar transcriptional reprogramming, affecting photosynthesis and stress responses. The common down-regulation of the ACD6 and FLS2 in both *gcn1* and *gcn20* mutants suggest that the observed defect in MAMP-induced stomatal closure of these two mutants could be mediated by these proteins.

Keywords: transcriptomic, chloroplast, translation, defence

Introduction

Selecting an mRNA for translation is a well-studied process requiring several consecutive steps and the contribution of more than 12 initiation factors. In virtually of eukaryotes, the majority of mRNAs initiate translation via a canonical cap-dependent mechanism, when the initiation factor eiF4E recognises a cap structure at the 5' end of the mRNA. Then, a cap-binding complex formed by eiF4E, eiF4G and eiF4A allow further recruitment of a ternary complex (eIF2/GTP/$tRNA^{met}$), the small ribosome subunit, and the additional initiation factors eIF3, eIF1 and eIF1A. This big complex then scans the mRNA until an ATG codon is found, after which the large ribosomal subunit is bound, and the elongation phase starts (Muñoz and Castellano, 2012).

Although most of the plant translation machinery resembles that of other eukaryotes, differences found in recent years in some of the components of this important process suggest that plants regulate their translation in unique ways. (Browning, 2004). Plants, for example, present a second eIF4F factor, called eIF(iso)4F, and certain features in the mRNAs allow different transcripts to interact with one or the other factor (Mayberry et al., 2009). In *Arabidopsis*, the double mutant of the two existing eIF(iso)4F isoforms show strong developmental phenotypes, (Lellis et al., 2010). General inhibition of translation is absence in this mutant, indicating that these factors are probably required for appropriate expression of specific genes that may participate in the regulation of plant growth and development.

The most puzzling mechanism for regulation of translation in plants is the one ruled by the phosphorylation of the eIF2α factor by specific kinases. This phosphorylation step prevents the interaction of eIF2-GDP to the eIF2B factor to regenerate GTP, thus blocking protein synthesis. Simultaneously, at least in yeast and mammals, a key transcription factor (GCN4 in yeast, ATF4 in mammals) is translationally derepressed when eIF2α is phosphorylated, to enhance expression of stress recovery genes. (Hinnebusch, 2005) This mode of regulation has been generally associated with cellular stresses, where general inhibition of the energy-consuming translation is needed in order to conserve resources and to initiate a reconfiguration of gene expression to effectively manage stress conditions (Castilho et al., 2014). Until recently, all the evidence indicated that this important mechanism of translational regulation could be also operating in plants. A functional eIF2α kinase (GCN2 for *General Control Non-repressive 2*) does exist in virtually all

plant genomes analysed, presenting all the structural domains of mammals or yeast eIF2α-kinases needed to perform its function. In fact, 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). Moreover, like mammals and yeast eIF2α-kinases, the *Arabidopsis* GCN2 protein interacts with uncharged tRNAs and has activity on different eIF2α isoforms (Li et al. 2013). However, although AtGCN2 phosphorylates eIF2α under many different stresses (Lageix et al., 2008), it has recently been shown that eIF2α phosphorylation does not correlate with global protein synthesis inhibition (Izquierdo et al 2018). This fact, together with the absence of GCN4 homologs, suggest that regulation of translation via eIF2α may be a minor pathways in plants.

The role of the GCN2-interacting proteins GCN1 and GCN20 is also elusive in plants. In yeast and mammals, activation of GCN2 requires binding to GCN1, forming a complex with the ATP-binding cassette protein GCN20, both attached to ribosomes. As a result, yeast *gcn1* and *gcn20* knock-out strains are deficient in GCN2 activation and eIF2α phosphorylation (Marton et al. 1993). In *Arabidopsis*, the only GCN1 homolog (also called ILITHYIA or NOXY7) (Monaghan et al., 2010; Izquierdo et al., 2018) also interacts with GCN2, and *gcn1* mutants are unable to phosphorylate eIF2α (Wang et al., 2016; Faus et al., 2018), foretelling a clear conservation of the mechanism. However, similarities stop there, as *gcn2* and *gcn1* mutants present very different phenotypes (Faus et al., 2018). Whereas *gcn2* mutants are indistinguishable from wild type, *gcn1* alleles present diverse developmental and stress-related defects, specially the stronger ones, (Monaghan et al., 2010; Faus et al., 2018), evidencing a GCN2-independent function for GCN1 in *Arabidopsis*, probably related also to the control of protein synthesis, as recently demonstrated by Izquierdo et al., 2018.

In yeast, GCN20 is another positive effector of GCN2, facilitating the activation of the kinase by uncharged tRNA on translating ribosomes. The N-terminal domain of GCN20 binds to the central eIF3-like domain of GCN1, thus modulating its activity (Marton et al., 1997; Garcia-Barrio et al., 2000). GCN20 belongs to a subfamily of ATP-binding cassettes without transmembrane domains, whose members are conserved among eukaryotes. There are 26 of those genes in Arabidopsis, five of them falling into a clearly differentiated cluster whose closest homolog in yeast is the GCN20 protein (Sanchéz-Fernández et al., 2001). One of those genes, *SCORD5* (for "*Susceptible to Coronatine (COR)-Deficient*"), was initially identified in a screen looking for Arabidopsis mutants that

could rescue the virulence of COR-deficient mutant bacteria (Zeng et al., 2011). SCORD5/GCN20 has been recently shown to interact with GCN1 in *Arabidopsis*; however, it is not essential for eIF2α phosphorylation (Izquierdo et al., 2018), and a common role with GCN1 in its GCN2-independent function has been suggested. Phenotypes of mutant-lines are also enigmatic. *scord5/gcn20* (henceforth *gcn20*) knock-out mutants present similar phenotypes to *gcn1* mutants. For example, both *gcn20* and *gcn1*, but not *gcn2*, show seedling yellowness and are unable to close stomata after bacterial infection (Zeng et al., 2011) and were sensitive to boric acid and antimycin A (Izquierdo et al., 2018). The three mutants, however, are susceptible to the amino acid synthesis inhibitor CHL, but only *gcn2* and *gcn20* are sensitive to paromomycin. These results indicate that the participation of the three GCN genes in translational regulation is far from being totally understood. Recently, we showed that GCN1 was involved in chloroplast biogenesis and root development, independent of GCN2 (Faus et al., 2018). In this work we describe phenotypic and molecular assays that reinforce the common function of GCN20 with GCN1 in biological processes where GCN2 seems not to be involved.

MATERIALS AND METHODS

Plant growth

The following genotypes were used in this study: wild-type Col-0, Col-7, *gcn1* (*ila3* (SALK_ 041123), *gcn2-2* (SALK_032196), *abcf1* (SAIL 412-A12), *abcf2* (SALK_018778C), *abcf3/gcn20* (*scord5-1* from Dr. Shang Yang He lab, Michigan State University, *abcf4* (SALK_202649C) and *abcf5* (SALK_113472C).

Seeds were pretreated in 70% ethanol for 20 min, surface-sterilized in 2.5% bleach for 5 min and washed with distillated water at least three times. After stratification at 4°C in the dark for 3 days, seeds were sown on 0,9% agar-containing 0,4% MS Salts, 1% sucrose, pH 5.7, and grown at 23°C with a 16-h-light/8-h-dark cycle.

For phenotyping at seedling stage, MS medium was supplemented with abscisic acid (0,8 μM), paraquat (0,7 μM), NaCl (125 mM) or acetic acid (4 mM) and percentage of seedlings with green cotyledons was calculated after 7-9 days. Three replicates containing 50 seeds per replicate was used for each treatment. Statistical differences between a genotype and its corresponding wild type were evaluated using a t-test. Differences with p-value lower than 0,05were considered significant.

For experiments in adult plants, 7-day-old plantlets were transferred to soil and 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. Visual inspection was followed during the next four weeks. Four trays containing 24 plants per tray and genotype were inspected. Representative pictures were taken for each phenotype.

P-eIF2α western blots

For these experiments, 10-days-old seedlings grown on MS media were used. Seedlings were UV-C exposed for around 25 minutes (9000 energy x2 + 4500. Stratalinker 1800) and collected immediately. A hundred seedlings per genotype were pooled, and protein was extracted using the P-EIF2α extraction buffer described elsewhere (Zhang et al. 2008). 20 µg 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 horseradich peroxidase-linked whole antibody (GE Healthcare) at a 1:10.000 dilution and visualized using a chemiluminescence system.

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 EtOH 30%-90% and incubated for 2h in LR-white resin in EtOH 90%, LR-white resin in EtOH 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). More than 30 images per genotype were inspected.

Microarray experiments

Total RNA was extracted from 20-days-old Col7 and abcf3/gcn20 plants. Transcriptome analysis was done using the Agilent *Arabidopsis* (V4) Gene Expression Agilent 4x44 Microarray. Three biological replicates of abcf3/gcn20 versus Col-7 wild-type comparisons were performed. Each biological replicate contained a pool of hundred seedlings per genotype. RNA integrity was assessed using the 2100 Bioanalyzer (Agilent). 0.5 µg RNA was amplified and labeled with the Agilent Low Input Quick Amp Labeling Kit. An Agilent Spi-

ke-In Kit was used to assess the labeling and hybridization efficiencies. Hybridization and slide washing were performed with the Gene Expression Hybridization Kit and Gene Expression Wash Buffers, respectively. After washing and drying, slides were scanned in a GenePix 4000B microarray scanner, at 5 μm resolution and using the double scanning. Image files were analyzed with the Feature Extraction software 9.5.1. Interarray analyses were performed with GeneSpring 11.5 software. To ensure a high-quality data set, control features were removed, and only those for which the 'IsWellAboveBG' parameter was 1 in at least two out of three replicates were 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 Enrichment analysis on Gene Ontology tools was performed using Agrigo v2.2 after Fisher test and multiple test correction (Tian et al., 2017), and a representative subset of the enriched GO-terms was obtained using a simple 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 numbers GSE136779

RESULTS

None of the five soluble *Arabidopsis* ABC transporters of the GCN subfamily is involved in eIF2α phosphorylation.

From the 26 ORFs of *Arabidopsis* encoding ABC proteins lacking contiguous transmembrane spans (Sanchéz-Fernández et al., 2001), the five members of the GCN subfamily fall into a clade based on sequence homology, with two nucleotide binding domains but without transmembrane spans. The five proteins share homology with the yeast GCN20 protein and other related proteins on the two ABC transporter domains, but present different N-terminal domains. GCN20 binds GCN1 in Arabidopsis but is not necessary for eIF2α phosphorylation (Izquierdo et al., 2018). We proceed to discard the possibility that any of the other GCN20-like proteins of the GCN ABC-transporters clade would be involved in GCN2 activation. With this purpose, T-DNA knock-out mutant lines of the five

gcn20-like genes were assayed for phosphorylation of the GCN2 substrate eIf2α. Seedlings were treated with UV-C light, known to phosphorylate eIf2α in *Arabidopsis* in a GCN2-dependent manner (Faus et al., 2018). As shown in Fig. 1, exposing *Arabidopsis* seedlings to UV-C stress results in a clear activation of GCN2 as detected by eIf2α phosphorylation on Western blots performed using phosphospecific antibodies for eIf2α (P-eIf2α). As reported by Izquierdo et al., 2018 phosphorylation is not avoided in the *scord5 (gcn20)* mutant allele. The other four mutant lines were also unable to phosphorylate eIF2α, suggesting that none of the five genes of the *Arabidopsis* GCN20 clade is essential for GCN2 activation.

GCN20 and GCN1, but not GCN2, share the same behavior under abiotic stresses.

To evaluate the consequence of the loss of function of the different GCN genes in the response to plant abiotic stress, we examined the phenotype of wild type and knock-out lines of gcn1(*ila3*), gcn2(*gcn2-2*), gcn20(*scord5*) and the other four gcn20-like genes under different abiotic conditions. As shown in Fig. 2, both *gcn1* and *gcn20* seedlings were clearly resistant to paraquat as compared to wild type, but no *gcn2* nor any of the other gcn20-like mutants (except *abcf5*). In contrast, only *gcn1* and *gcn20* were more sensitive than wild type to treatment with abscisic acid, whereas *gcn2* and the other four gcn20-like mutants behave similarly to wild type. Similarly, in response to NaCl, *gcn1* and *gcn20*, but no *gcn2* were more sensitive than wild type. In this case, the other four gcn20-like mutants behave differently, being *abcf1, abcf2* and *abcf5* more sensitive than wild type, and *abcf4* behaving similar to wild type. In medium supplemented with to acetic acid, however, only *gcn20* and *abcf1* presents sensitivity. In summary, these data suggest a coordinated behavior of GCN1 and GCN20 in response to most of these abiotic stresses, independent of the function of GNC2. The behavior of the other four gcn20-like genes is not correlated with gcn1 in most of the stresses assayed, and their functional link deserve further studies.

GCN20 and GCN1 share chloroplast defects

Contrary to *gcn2* plants, which are indistinguishable from wild type, *gcn1*plants are yellow to light green in colour, especially in emerging leaves (Monaghan et al., 2010). *gcn20* and the other gcn20-like mutants were grown and their phenotypes compared to *gcn1* and *gcn2*. As observed in Fig. 2, only *gcn20*, but not the other four knock-out gcn20-like mutants present yellow leaves, reinforcing the link between *gcn1* and *gcn20*, and discarding a role of any of the other gcn20-like genes in the developmental phenotypes ob-

served in the *gcn1* mutant. We also had showed that *gcn1* mutants, but not *gcn2,* present altered root and chloroplast development (Faus et al., 2018). Recently, it was reported that GCN20, similarly to GCN1, is also involved in root development, by modulating DNA damage repair (Han et al., 2018). In order to know if the chloroplast defects of *gcn1* were also shared by *gcn20,* electron microscopy experiments were performed. As shown in Fig. 3, thylakoid organization is severely affected in *gcn20* leaves. Wild type leaves contained fully developed chloroplasts with internal thylakoid membranes stacked into grana layers (Fig. 3a). In contrast, and similarly to *gcn1* chloroplasts (Fig. 3b), *gcn20* chloroplast contained a poorly developed thylakoid network with wide luminal areas between the thylakoid membranes (Fig. 3c). *gcn2* chloroplasts presented an appearance similar to wild type, with a well-established thylakoid structure and correctly stacked grana system (Faus et al., 2018). These results suggest that the GCN20 protein is necessary for the correct development of the thylakoid network in the chloroplasts, a role that could be performed together with GCN1 but independent of GCN2.

gcn20 and _gcn1_ mutant plants share transcriptomic profiles.

In order to examine to what extend the consequences of gcn20 loss-of-function are shared by the loss-of–function of the gcn1 gene, microarray experiments comparing gene expression of two-weeks old wild type and *gcn20/scord5* seedlings were performed, and differentially expressed genes were contrasted to those reported by Faus et al., 2018 after comparing wild type versus *gcn1/ila3* seedlings of the same age. Seedlings of wild type and *gcn2* of the same age do not differ in their transcriptomic profiles (Faus et al., 2015). Following the same criteria as in Faus et al., 391 genes were considered upregulated and 215 downregulated in the *gcn20/scord5* mutant (Supplemental table 1). Defense response and photosynthesis-related categories were enriched upon downregulated genes in *gcn1/ila3* (Faus et al., 2018). Gene enrichment analysis upon the genes downregulated in *gcn20/scord5* also identified categories related to defense, such as defense response (FDR $1.64e^{-16}$), or more specifically, incompatible interaction (FDR $7.1e^{-06}$) or response to salicylic acid (SA) (FDR $6.26e^{-08}$), and photosynthesis, light reaction (FDR, 0.03), indicating that loss-of-function of any of the two genes compromises similar biological processes (Supplemental table 2, Fig. 4a). Indeed, as shown in Fig. 4b, more than 50% of the genes downregulated in *gcn20/scord5* were also downregulated in *gcn1/ila3*. Among the genes downregulated in *gcn20/scord5*, we found several cysteine-rich receptor-like protein kinases (CRKs), playing relevant roles in the regulation of pathogen defense and programmed cell death, ALD1, involved in pipecolin acid produc-

tion, relevant for systemic acquired resistance (SAR) signaling, the SAR-marker PR1, the PTI marker FRK1 or several WRKY transcription factors involved in defense responses. Similarly, photosystem II-related genes such as LHB1B1, Lhcb2.4, or the chlorophyll binding protein D1, a part of the reaction center PSBA, as well as the FED A major leaf ferredoxine, were also downregulated in *gcn20/scord5*. (Supplemental table 1). Upon the categories enriched in *gcn20/scord5* upregulated genes are response to chitin (FDR, $1.6e^{-14}$), response to heat (FDR, 0.9 e^{-4}), and response to oxidative stress (FDR, 0.0014), (Supplemental table 2, Fig. 4a) all coincident with those observed in *gcn1/ila3* analysis. Accordingly, 33% of the genes were overexpressed in both mutants. However, new categories were observed enriched only in the *gcn20/scord5* overexpressed genes, including regulation of transcription (FDR, $1.6e^{-09}$), or response of jasmonic acid (FDR, 0.1 e^{-4}) among others. All these results clearly indicate that *gcn20/scord5* loss-of-function alters the transcriptomic profile of the plant in many processes shared by *gcn1/ila3*, but also in others not previously observed in this mutant.

A very well detailed function for GCN20 is that this protein is required for bacterium-triggered stomata closure response. The *gcn20/scord5* mutation affected MAMP-induced stomatal closure, but not SA- or ABA-induced stomatal closure, suggesting that GCN20 likely acts early in the stomatal closure response pathway. Among the genes that are down-regulated in both mutants, we found *ACD6*. This gene was nearly four times repressed in *gcn20/scord5*, and also in *gcn1/ila3*. *ACD6* positively controls the membrane levels of the flagellin receptor FLS2 (Tateda et al. 2014, Zhang et al. 2014), essential for stomatal response (Zheng et al., 2010). In addition, the cysteine-rich receptor-like protein kinase CRK4 was found 5.5 times less expressed in *gcn20/scord5*, and it was found 3.2 times less expressed in *gcn1/ila3*. CRK4 interacts with FLS2, and it has been described that CRK4 overexpression lines present enhanced stomatal immunity (Yeh et al., 2016). These results could indicate that the role of SCORD5 and ILA3 in stomata closure response could be through the regulation of FLS2 levels.

DISCUSSION

Initially identified in yeast as a suppressor mutation that overcomes the toxic effect of a constitutive GCN2 allele, the GCN20 protein is a positive effector of the eIF-2a kinase activity of GCN2, and it forms, together with GCN1, a protein complex required for the activation of GCN2 by uncharged tRNA on translating ribosomes (Marton et el, 1997). The whole system is an important hub controlling mRNA translation and stress adaptation in

yeast, *C. elegans* or mammals (Hirose and Horvitz, 2014), and the conservation of these three proteins initially suggested a conserved regulatory mechanism in plants. However, recent results obtained in *Arabidopsis* indicate a minor relevance of GCN2 and P-eIF2α in the overall inhibition of translation (Izquierdo et al., 2018): in essence, eIF2α is phosphorylated by GCN2 in many stress situations, but the exact role of this phosphorylation is still unknown. Mechanistically, GCN1 is required for eIF2α-phosphorylation in *Arabidopsis*, but unexpectedly, SCORD5, a protein homologous to the yeast GCN20, is not (Izquierdo et al., 2018). We also discarded that loss-of-function of the other GCN20-like isoforms could compromise eIF2α-phosphorylation. The five members of the GCN20 subfamily in *Arabidopsis* contain two ABC domains without a transmembrane domain but differ markedly in the N-termini. Non-membrane ABC proteins are known to be involved in translation. The human ABC50 protein, for instance (the only member of the ABCF family, apart from GCN20, which has been characterized in detail), binds to ribosomes and interacts with the eukaryotic initiation factor eIF2, which plays a key role in translational initiation control. Moreover, since it is only the N-terminal of GCN20 that is required to support the function of GCN2 in yeast (Marton et al., 1997), and the five *Arabidopsis* ABCF proteins differ in their N-termini, it seems unlikely that this phenotype is explained by gene redundancy. Either GCN1 can perform GCN2 activation without the help of GCN20, or another protein is performing this role in *Arabidopsis*. On the other hand, GCN1 interacts with GCN20, and they coordinately regulate stress responses through translational regulation, but independent of GCN2 (Izquierdo et al., 2018). The hypothesis that GCN1 could have a GCN2-independent function is reinforced by the different phenotype of *gcn2* and *gcn1* loss-of-functions alleles. Whereas *gcn2* plants are indistinguishable from wild type, *gcn1* alleles present clear stress-related and developmental phenotypes (Monaghan et al., 2010; Faus et al., 2018). Curiously, as initially reported by Zeng et al., 2011, *gcn1* and *gcn20* alleles are unable to close stomata after bacterial infection, and are susceptible to pathogen attack. Later, Izquierdo et al., 2018 wider the similarities between these two proteins by examining their behavior after boric acid stress, and suggested a coordinated role of these two proteins in protein synthesis. Here, the similar behavior after paraquat, NaCl and abscisic acid treatments and the similar defects in chloroplast development further reinforces a coordinated action of GCN1 and GCN20 in response to environmental stresses, independent of the action of GCN2. Tolerance to paraquat, a potent superoxide producer, could be explained as the category “response to oxidative stress” was highlighted upon the gene upregulated in *gcn20* (*scord5),* as it happened with *gcn1* (*ila3*)(Faus et al., 2015). In particular, the

chloroplastic copper/zinc superoxide dismutase SOD1 was three-fold induced in *gcn1*, and SOD2 was two-fold induced in *gcn20*. However, since chloroplast is impaired in the two mutants, electron transport should be weakened as well. Thus, the capacity of the mutants to generate ROS from paraquat should have been reduced. It is hard to conclude what is the cause of tolerance without further evidence. The fact that loss of function of any of these two proteins confers tolerance to paraquat but susceptibility to NaCl is intriguing, as oxidative stress is an important component involved in salinity-induced damage (Moradi and Ismail 2007), and the accumulation of NaCl is followed by an increase of superoxide and H_2O_2 (Mishra et al., 2013). Considering that both mutants are sensitive to ABA, that mimics osmotic stress, we suggest that the osmotic component of NaCl toxicity could be the cause of the observed phenotypes.

Transcriptomes of *gcn20* and *gcn1* support other previously reported phenotypic results. Defects in chloroplast biogenesis (Faus at el, 2018) is evidenced by the down-regulation of genes related to photosynthesis and the similar defects observed in *gcn20* and *gcn1* chloroplasts. The increased expression in *gcn1* tissues of genes involved in removal of superoxide could indicate a context of oxidative stress in these mutants. Mutants in the ferredoxin-NADP(+)-oxidoreductase gene, that also display a highly chloroplast-deficient phenotype also induce ROS-scavenging systems to protect damaged chloroplasts. (Lintala et al., 2012). A similar mechanism could also be occurring in *gcn1* and *gcn20* leaves as a common response to protect defective tissues that result from the mutation.

Also, defects in immunity (Monaghan et al., 2010) relates with the shared down-regulation of genes involved in defense responses, mainly in salicylic-related responses. Very interestingly, these include the ACD6 and the CRK4 genes, two genes whose down-regulation is known to directly affect the function of the FLS2 receptors. *gcn1* and *gcn20* mutants share their impossibility to close stomata after bacterial infection (Zheng et al., 2011). This response is triggered by the well-characterized pathogen-associated molecular pattern (PAMP) flg22 (a peptide derived from bacterial flagellin). Flagellin (o flg22) is recognized in the plant cell by the flagellin-receptor FLS2, which activates the signaling cascade involved in pathogen- or PAMP-induced stomatal closure (Zeng et al., 2010). A major function for ACD6 is to regulate the plasma membrane pool of FLS2 (Zhang et al., 2014). ACD6 and FLS2 form a protein complex in the plasma membrane, and in benzothiadiazole-treated plants that lacked ACD6 (*acd6-2* mutants), FLS2 failed to show increased levels in this compartment. A role for ACD6 in FLS2-mediated signaling is also suggested by the reduced transcriptional response of the *acd6-2* loss-of-function mutant

to the FLS2 ligand flg22. FLS2 pools and downstream responses could be also diminished in *gcn20* or *gcn1* plants, in which ACD6 is downregulated. In addition, it has been reported that overexpression of cysteine-rich receptor-like kinase 4 (CRK4) present a defective Pst DC3000-mediated stomatal reopening (Yeh et al., 2015), indicating a role of this gene early during the activation of this PTI response. Co-IP assays indicated that CRK4 associates with FLS2, although in this case the mechanisms are unknown. The down-regulation of CRK4 levels in *gcn20* and *gcn1* mutants could again affect the proper function of the FLS2-mediated stomatal closure after infection.

The response to jasmonic acid was enriched in the *gcn20* overexpressed genes, and this could also explain the repression of the salicylic responses observed in this mutant. The antagonistic effect of jasmonic acid (JA) and salicylic pathways is well documented. Some pathogens disable SA signalling, as the bacterial toxin coronatine (COR) mimics JA. COR can activate the JA signaling pathway, and enhance bacterial virulence by suppressing SA-mediated defence through hormonal crosstalk. (Kazan and Lyons, 2014). The higher expression in *gcn20* mutants of the MYC2 transcription factor, that activates the jasmonic acid pathway and is involved in the salicylic acid crosstalk (Zheng et al., 2012; Du et al., 2017)), could be responsible of the inhibition of the salicylic pathway and the lower expression of defence genes in *gcn20*.

In summary, phenotypic and molecular data further confirm the functional association of GCN1 and GCN20 in a GCN2-independent manner and reinforces the idea of a new function for these two proteins. The assays of *gcn1* and *gcn20* mutants after Pst DC3000 infection (Izquierdo et al., 2018) supports a role for GCN1 and GCN20 in the preinvasive response to bacterial infection, in which these proteins could regulate translation of specific proteins. However, the basal transcriptomic profiles of both mutants indicate that this level of regulation could also be determinant for this phenotype.

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Conflict of Interest Statement

The authors declare no conflict of interest.

REFERENCES

Browning KS. (2004). Plant translation initiation factors: it is not easy to be green. Biochem Soc Trans. 32(Pt 4):589-91.

Castilho BA, Shanmugam R, Silva RC, Ramesh R, Himme BM, Sattlegger E. (2014). Keeping the eIF2 alpha kinase Gcn2 in check. Biochim Biophys Acta. 1843(9):1948-68.

Du M, Zhao J, Tzeng DTW, Liu Y, Deng L, Yang T, Zhai Q, Wu F, Huang Z, Zhou M, Wang Q, Chen Q, Zhong S, Li CB, Li C. (2017). MYC2 Orchestrates a Hierarchical Transcriptional Cascade That Regulates Jasmonate-Mediated Plant Immunity in Tomato. Plant Cell.29(8): 1883-1906.

Faus I, Niñoles R, Kesari V, Llabata P, Tam E, Nebauer SG, Santiago J, Hauser MT, Gadea J. (2018). *Arabidopsis* ILITHYIA protein is necessary for proper chloroplast biogenesis and root development independent of eIF2α phosphorylation. J Plant Physiol. May – Jun 224-225:173-182.

Faus I, Zabalza A, Santiago J, Nebauer SG, Royuela M, Serrano R, Gadea J. (2015) Protein kinase GCN2 mediates responses to glyphosate in *Arabidopsis*. BMC Plant Biol. 15:14.

Garcia-Barrio M, Dong J, Ufano S, Hinnebusch AG. (2000) Association of GCN1-GCN20 regulatory complex with the N-terminus of eIF2alpha kinase GCN2 is required for GCN2 activation. EMBO J. 19(8):1887-99.

Han TT, Liu WC, Lu YT. (2018) General control non-repressible 20 (GCN20) functions in root growth by modulating DNA damage repair in *Arabidopsis*. BMC Plant Biol. 18(1):274.

Hinnebusch A.G. (2005) Translational regulation of GCN4 and the general amino acid control of yeast Annu. Rev. Microbiol., 59: 407-450

Hirose T, Horvitz HR. (2014) The translational regulators GCN-1 and ABCF-3 act together to promote apoptosis in C. elegans. PLoS Genet. 10(8): e1004512.

Izquierdo Y, Kulasekaran S, Benito P, López B, Marcos R, Cascón T, Hamberg M, Castresana C. (2018). *Arabidopsis* nonresponding to oxylipins locus NOXY7 encodes a yeast GCN1 homolog that mediates noncanonical translation regulation and stress adaptation. Plant Cell Environ. 41(6):1438-1452.

Kazan K, Lyons R. (2014) Intervention of Phytohormone Pathways by Pathogen Effectors. Plant Cell. 26(6):2285-2309.

Lageix S, Lanet E, Pouch-Pélissier MN, Espagnol MC, Robaglia C, Deragon JM, Pélissier T. (2008). *Arabidopsis* eIF2alpha kinase GCN2 is essential for growth in stress conditions and is activated by wounding. BMC Plant Biol. 8:134.

Lellis AD, Allen ML, Aertker AW, Tran JK, Hillis DM, Harbin CR, Caldwell C, Gallie DR, Browning KS. (2010). Deletion of the eIFiso4G subunit of the *Arabidopsis* eIFiso4F translation initiation complex impairs health and viability. Plant Mol Biol. 74(3):249-63.

Li MW, AuYeung WK, Lam HM. (2013). The GCN2 homologue in *Arabidopsis thaliana* interacts with uncharged tRNA and uses *Arabidopsis* eIF2α molecules as direct substrates. Plant Biology (Stuttgart), 15(1):13-18.

Lintala M, Lehtimäki N, Benz JP, Jungfer A, Soll J, Aro EM, Bölter B, Mulo P. (2012) Depletion of leaf-type ferredoxin-NADP(+) oxidoreductase results in the permanent induction of photoprotective mechanisms in *Arabidopsis* chloroplasts. Plant J. 70(5):809-17.

Marton M.J., Crouch D. & Hinnebusch A.G. (1993). GCN1, a translational activator of GCN4 in Saccharomyces cerevisiae, is required for phosphorylation of eukaryotic translation initiation factor 2 by protein kinase GCN2. Molecular and Cellular Biology. 13(6): 3541-56.

Marton MJ, deAldana CRV, Qiu HF, Chakraburtty K, Hinnebusch AG. (1997). Evidence that GCN1 and GCN20, translational regulators of GCN4, function on elongating ribosomes in activation of eIF2 alpha kinase GCN2. Molecular and Cellular Biology, 17(8):4474-4489.

Mayberry LK, Allen ML, Dennis MD, Browning KS. (2009). Evidence for variation in the optimal translation initiation complex: plant eIF4B, eIF4F, and eIF(iso)4F differentially promote translation of mRNAs. Plant Physiol. 150(4):1844-54.

Mishra P, Bhoomika K, Dubey RS. (2013). Differential responses of antioxidative defense system to prolonged salinity stress in salt-tolerant and salt-sensitive Indica rice (Oryza sativa L.) seedlings. Protoplasma. 250(1):3-19.

Monaghan J, Li X. (2010). The HEAT Repeat Protein ILITYHIA is Required for Plant Immunity. Plant and Cell Physiology, 51(5):742-753.

Moradi F, Ismail AM. (2007). Responses of photosynthesis, chlorophyll fluorescence and ROS-scavenging systems to salt stress during seedling and reproductive stages in rice. Ann Bot. Jun;99(6):1161-73.

Muñoz A, Castellano MM. (2012). Regulation of Translation Initiation under Abiotic Stress Conditions in Plants: Is It a Conserved or Not so Conserved Process among Eukaryotes? Comp Funct Genomics. 2012:406357.

Sánchez-Fernández R, Davies TG, Coleman JO, Rea PA. (2001). The *Arabidopsis thaliana* ABC protein superfamily, a complete inventory. J Biol Chem. 276(32):30231-44.

Supek F., Bošnjak M., Škunca N. & Šmuc T. (2011) REVIGO summarizes and visualizes long lists of gene ontology terms. PLoS One 6(7): e21800.

Tateda C, Zhang Z, Greenberg JT. (2015). Linking pattern recognition and salicylic acid responses in *Arabidopsis* through ACCELERATED CELL DEATH6 and receptors. Plant Signal Behav. 10(10): e1010912.

Tian T, Liu Y, Yan H, You Q, Yi X, Du Z, Xu W, Su Z.(2017) AgriGO v2.0: a GO analysis toolkit for the agricultural community, 2017 update. Nucleic Acids Res. 45(W1): W122-W129. doi: 10.1093/nar/gkx382.

Tusher V.G., Tibshirani R. & Chu G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A.98 (9), 5116-21.

Yeh YH, Chang YH, Huang PY, Huang JB, Zimmerli L. (2015). Enhanced *Arabidopsis* pattern-triggered immunity by overexpression of cysteine-rich receptor-like kinases. Front Plant Sci. 6:322.

Wang L, Li H, Zhao C, Li S, Kong L, Wu W, Kong W, Liu Y, Wei Y, Zhu JK, Zhang H. (2016). The inhibition of protein translation mediated by AtGCN1 is essential for cold tolerance in *Arabidopsis thaliana*. Plant Cell and Environment 40(1):56-68.

Zeng W, Melotto M, He SY. (2010) Plant stomata: a checkpoint of host immunity and pathogen virulence. Curr Opin Biotechnol.21(5):599-603. doi: 10.1016/j.copbio. 2010.05.006. Epub 2010 Jun 21.

Zeng W, Brutus A, Kremer JM, Withers JC, Gao X, Jones AD, He SY. (2011). A Genetic Screen Reveals Arabidopsis Stomatal and/or Apoplastic Defenses against Pseudomonas syringae pv. tomato DC3000. Plos Pathogens, 7(10).

Zhang Y, Dickinson JR, Paul MJ, Halford NG: (2003). Molecular cloning of an *arabidopsis* homologue of GCN2, a protein kinase involved in co-ordinated response to amino acid starvation. Planta 217(4):668-675.

Zhang Y., Wang Y., Kanyuka K., Parry M.A., Powers S.J., & Halford N.G. (2008). GCN2-dependent phosphorylation of eukaryotic translation initiation factor-2alpha in *Arabidopsis*. Journal of Experimental Botany 59(11):3131-41.

Zhang Z, Shrestha J, Tateda C, Greenberg JT. (2014). Salicylic acid signaling controls the maturation and localization of the *arabidopsis* defense protein ACCELERATED CELL DEATH6. Mol Plant. 7(8):1365-1383.

Zheng, X. Y., Spivey, N. W., Zeng, W., Liu, P. P., Fu, Z. Q., Klessig, D. F.et al. (2012). Coronatine promotes Pseudomonas syringae virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. Cell host & microbe 11(6), 587–596. doi:10.1016/j.chom.2012.04.014

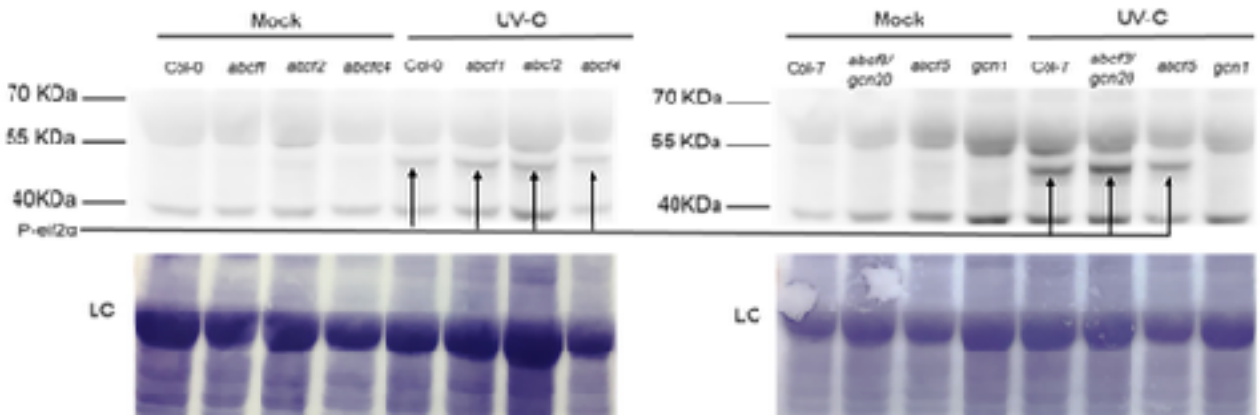

Fig. 1. Western blot assaying eIF2α phosphorylation on wild-type (Col-0 or Col-7), *abcf1,2,3,4* and *5* mutants and *gcn1* (*ila-3*) mutant seedlings, mock-treated and treated with UV-C to induce phosphorylation. A differential band corresponding to P-eIF2α is shown by arrows. Equal amount of protein (20µg) was loaded in a 10% SDS-PAGE gel. LC: Loading control.

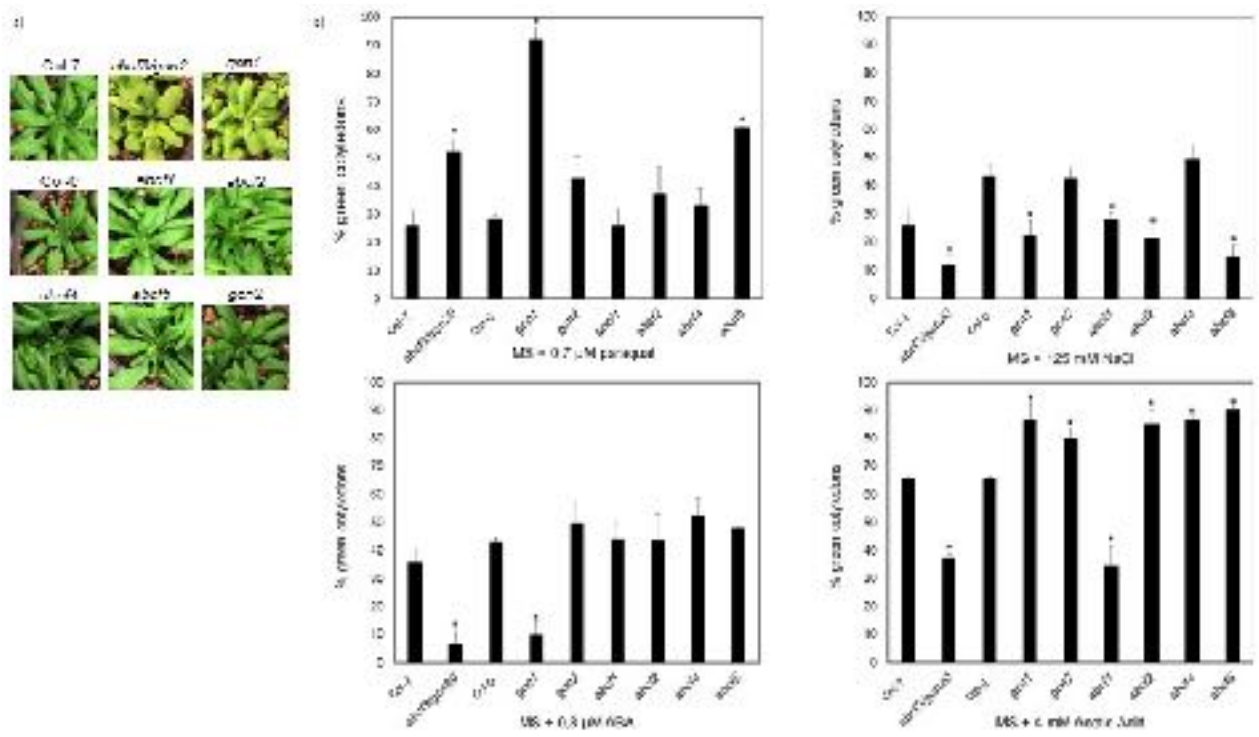

Fig. 2. gcn20 and gcn1 mutants share similar phenotypes in normal and stress conditions. a) Representative image of the rosette leaves of wild type (Col-0 or Col-7), abcf1,2,3,4

and 5, gcn1 (ila-3) and gcn2 (gcn2-2) plants after growing 4 weeks in the greenhouse. Four trays containing 24 plants per tray and genotype were prepared. Representative pictures were taken for each phenotype. b) Percentage of seedlings with green cotyledons after growing 7 days on MS medium supplemented with 0,7 µM paraquat (top left), 125 mM NaCl (top right) or 4 mM acetic acid (bottom right) and after growing 9 days on MS medium supplemented with 0,8 µM ABA (bottom left). 100% green cotyledons were obtained in all genotypes sowed in MS plants. Bars represent mean and standard error of three biological replicates. * indicates statistical differences after t-test (p-value= 0,05) between a genotype and its corresponding wild type.Three replicates containing 50 seeds per replicate was used for each treatment.

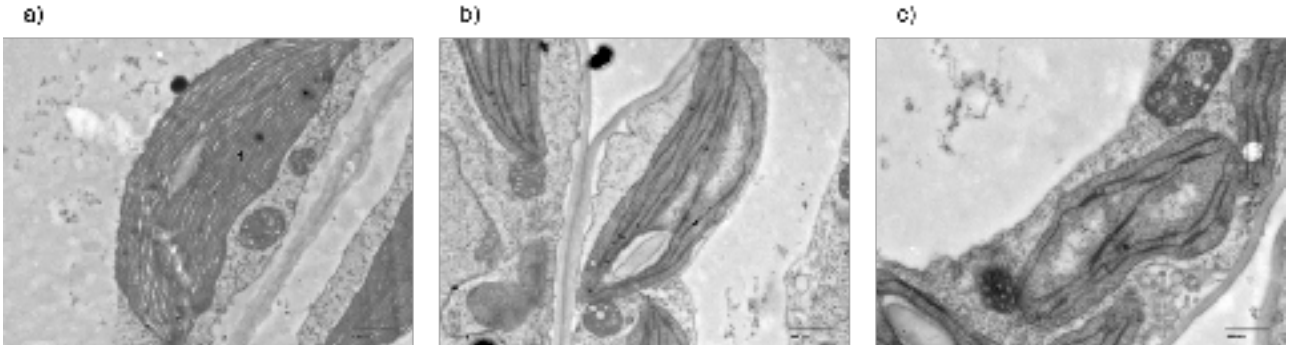

Fig. 3. *gcn20* mutant has defective chloroplast development. Transmission electron microscopy images of Col-0 (a), *gcn1 (ila3)* (b), and *gcn20* (d) leaves chloroplasts showing internal thylakoid membranes. Plants were grown for 30 days in the greenhouse under long-day conditions. Scale bars: 800 nm (a,b) or 500 nm (c).

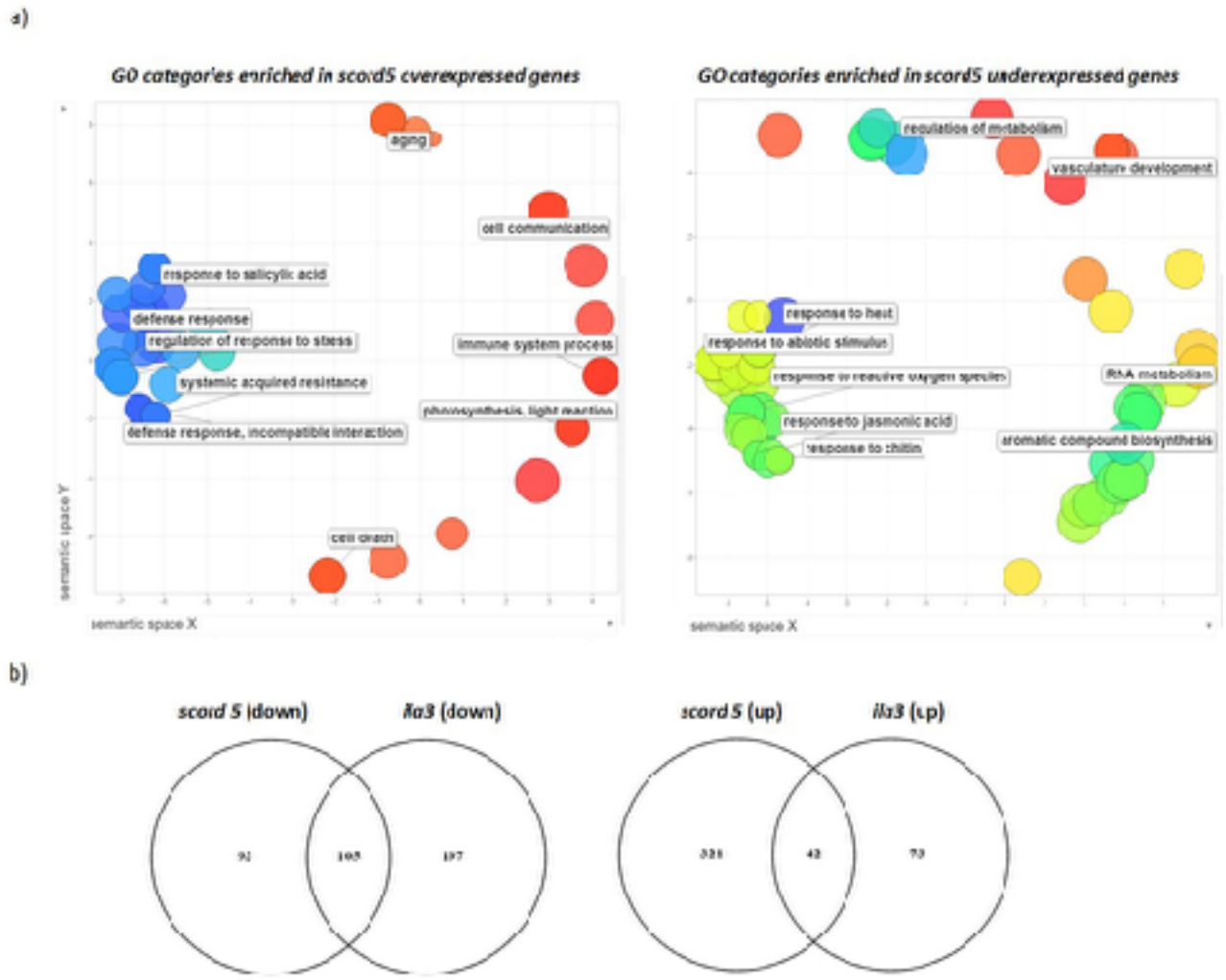

Fig. 4. *gcn20* and *gcn1* mutant plants share transcriptomic profiles. a) Representative GO categories enriched in *gcn20* overexpressed and underexpressed genes. The scatter-plot shows the cluster representatives (terms remaining after the redundancy reduction) in a two dimensional space derived by applying multidimensional scaling to a matrix of the GO terms' semantic similarities, according to REVIGO software. Color scale (log10 p-value). b) Venn diagrams showing number of genes overexpressed and underexpressed in *ila3* (Faus et al., 2018) and *gcn20 (scord5)*

Supplemental Table 1 Differentially-expressed genes in the transcriptomic experiment comparing wild-type and *gcn20* seedlings. Columns correspond to: B. numerator after SAM test (average fold-change) C. q-value D-F. Independent ratios gcn20/wild-type in the three replicates

Supplemental Table 2 Gene ontology categories considered enriched after Fisher test, and multiple test correction according to Agrigo v2.2, among the genes differentially expressed in the transcriptomic experiment comparing wild-type and gcn20 seedlings.

4.DISCUSIÓN

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4.1. Importancia del sistema GCN2 en células eucariotas

Las células eucariotas presentan mecanismos de regulación que les permiten ajustarse al ambiente que les rodea y reajustar así su metabolismo para un óptimo funcionamiento. Por ejemplo, ante situaciones de estrés, las condiciones óptimas se ven alteradas, y las células activan diferentes mecanismos de reajuste molecular para combatir estos estreses, tanto bióticos como abióticos. Uno de los principales procesos celulares que se regulan ante situaciones de estrés es la traducción. Este proceso comprende un conjunto de reacciones biosintéticas que necesitan altas cantidades de energía, por lo que, ante situaciones de estrés, un sistema muy conservado de control es la paralización de la traducción, para conseguir un ahorro energético en la célula. Además, dicha regulación de la traducción se encuentra también implicada en la activación selectiva de respuestas apropiadas para superar las diferentes situaciones de estrés celular.

La proteína quinasa eIF2α es una de las proteínas que regulan la traducción en células eucariotas durante situaciones de estrés celular (de Haro et al., 1996), provocando la inhibición generalizada de la síntesis de proteínas y la activación de la traducción de determinados mRNA que codifican factores celulares implicados en la respuesta a estrés (Dever et at., 1992; Harding et at., 2000; Jackson et at., 2010). GCN2 es una proteína quinasa que fosforila el factor de iniciación eIF2α, conservada en células eucariotas desde levaduras hasta mamíferos. Su papel principal es parar la traducción mediante la fosforilación del factor eIF2α, y activar la traducción selectiva de los factores de transcripción GCN4 en levaduras y ATF4 en mamíferos. GCN2 se activa a través de tRNAs no cargados en respuesta al ayuno de aminoácidos. Este sistema regulador es importante para tolerar la privación de nutrientes y otros estreses, y mantener un correcto desarrollo, diferenciación y función de los órganos en los mamíferos. Además, en mamíferos, se sabe que no solo se activa frente al estrés por ayuno de aminoácidos, como ya se ha comentado varias veces en la Introducción, sino que también se activa ante la exposición a luz UV (Deng et al., 2002; Hinnebusch., 2005) y en presencia de RNA viral (Berlanga et al., 2006).

En los últimos años, la importancia de un sistema de control de la traducción en animales como el sistema GCN, ha quedado revelado por la implicación de GCN2 en aspectos

como la modulación de la esperanza de vida, la supervivencia de las células tumorales y las respuestas inmunes (Murguía y Serrano et al., 2012). Por ejemplo, un microambiente tumoral se caracteriza por un bajo flujo sanguíneo, el cual desencadena la privación de nutrientes al tumor y la hipoxia del mismo. Las células cancerosas sobreviven a estas situaciones de estrés mediante la activación de GCN2 y su objetivo, el factor ATF4. ATF4 está implicado en la absorción de nutrientes y la tolerancia al estrés oxidativo, mejorando así la supervivencia de las células tumorales (Wek et al., 2006). Hay estudios que demostraron que la inactivación del factor de transcripción ATF4 provoca la muerte de las células tumores por parada del ciclo celular y apoptosis. Por otro lado, la activación de GCN2 por ayuno de aminoácidos en las células tumores parece estar asociado al agotamiento de la asparagina, ya que un exceso de este aminoácido alivia la muerte celular inducida por la pérdida de ATF4 y la caída de ATF4 reduce la expresión de la asparagina sintetasa (Ye et al., 2010). En otro estudio se demuestra que los mecanismos de detección de inanición de aminoácidos mediados por GCN2 pueden condicionar la inflamación intestinal en ratones con sulfato de sodio dextrano (DSS), lo que indica que GCN2 tiene la capacidad de modular las respuestas inmunes, especialmente la inflamación (Xia et al., 2018). Finalmente, a la ruta clásica de respuesta a ayuno de nutrientes se han añadido recientemente nuevas funciones para GCN2, modulando la traducción de nuevos mRNA como los de un regulador del transporte de leucina y de la sulfiredoxina SRX1 en levadura (activación de la traducción) o la sintasa inducible de óxido nítrico, ErBb2, HIF1a y mRNA del tracto de la oligopirimidina 5'-terminal en mamíferos (inhibición de la traducción). También se ha visto que GCN2 es capaz de fosforilar nuevas proteínas como la metionil-tRNA sintetasa en mamíferos, una vía para la reparación del DNA (Murguía y Serrano et al., 2012). Estos ejemplos ilustran la relevancia que el sistema de regulación mediado por esta quinasa estaba teniendo en los últimos años en el conocimiento de los mecanismos celulares de homeostasis en células eucariotas no vegetales. El sistema de control de la traducción a través de eIF2α en células animales no sólo implica a GCN2, sino a otras tres quinasas, lo que refuerza su papel clave en la regulación celular. Se ha visto que GCN2 es uno de los cuatro sensores conocidos de la respuesta al estrés integrada (ISR). La ISR es una respuesta citoprotectora mediada por eIF2α que permite a las células detectar y responder a diferentes señales celulares, incluido el estrés del retículo endoplásmatico, proteínas desplegadas y privación de nutrientes.

El año que comenzamos este trabajo, pocos eran los datos que se tenían sobre la presencia de estas quinasas en plantas. En *Arabidopsis s*e había descubierto una proteína homóloga que complementaba al mutante GCN2 de levadura, lo cual indicaba que la respuesta reguladora GAAC (control general de aminoácidos) que se produce en levaduras, podría estar dándose también en plantas (Zhang et al., 2003). En plantas de *Arabidopsis* con la biosíntesis de aminoácidos deteriorada tras un tratamiento con glifosato, eIF2α es fosforilada y esta fosforilación depende únicamente de GCN2, indicando que en genomas vegetales una única quinasa podría estar controlando la respuesta a estrés integrada (Zhang et al., 2008). En los últimos años, la fosforilación de eIF2 por GCN2 se ha observado ya en presencia de diferentes estreses abióticos tales como el ayuno de aminoácidos y purinas, luz UV, choques térmicos de frío y heridas (Zhang et al., 2008; Lageix et al., 2008). Además se ha visto una parada transcripcional dependiente de GCN2 después de tratamientos con clorsulfuron, un inhibidor de la biosíntesis de aminoácidos ramificados. Estos tratamientos parecían indicar que GCN2 (AtGCN2) era relevante para el crecimiento de las plantas en situaciones de estrés y que su actividad podría dar como resultado una descenso en la síntesis global de proteínas en multitud de situaciones (Lageix et al., 2008).

A pesar de estos datos prometedores, y tras varios años de nuevos resultados por nuestro grupo en el transcurso de esta tesis y por otros grupos, la importancia de la vía GCN2 en plantas como mecanismo regulador aún está en debate (Immanuel et at., 2012). En plantas no se ha encontrado homólogo de *GCN4*, gen importante de este mecanismo en levaduras; además existe poca evidencia de la implicación de AtGCN2 en la regulación de la expresión de los genes de la biosíntesis de aminoácidos, lo que sugiere que esta quinasa podría estar desempeñando un papel diferente al de la regulación de la biosíntesis de estos compuestos (Byrne et al., 2012). Los estudios realizados hasta el momento de comenzar este trabajo así como durante el mismo, dejaban entrever que se conserva una respuesta general del control de aminoácidos entre las levaduras y las plantas, pero que la enzima vegetal en algún momento evolucionó para cumplir una función más general como sensor y regulador de diversas vías de respuesta al estrés en plantas. Lageix y sus colaboradores demostraron la activación de AtGCN2 después de una herida o exposición al metil-jasmonato, al precursor de etileno ácido 1–aminociclopropano–1–carboxílico (ACC) y al ácido salicílico, lo que sugiere que esta enzima además, podría desempeñar un papel en la defensa de las plantas contra los insectos herbívoros (Lageix et al., 2008). Sin embargo, como se verá más adelante, la relevancia de este sistema de control en

plantas sigue sin estar clara, y la consecuencia en la planta al no poder activar eIF2α mediante mutantes de pérdida de función no parece indicar que la activación de GCN2 vaya ligada a una mejor respuesta ante condiciones de estrés.

4.2. El sistema GCN en *Arabidopsis thaliana*

En vertebrados, se sabe que cuatro quinasas diferentes fosforilan eIF2α (Hinnebusch et al., 2005). Por otro lado, únicamente las plantas y las levaduras presentan tan solo una, la quinasa GCN2. Como se ha comentado, en situaciones de estrés, las proteínas eIF2α quinasas se activan, fosforilan al factor eIF2, provocan la inhibición generalizada de la traducción y activan la traducción del mRNA de factores implicados en la respuesta a estrés. La existencia de GCN2 en plantas, cuya secuencia presenta una homología del 45% frente al gen de levadura (Zhang et al., 2003), sugiere que la ruta dependiente de GCN para la fosforilación de eIF2α se conserva en las plantas. Algunos de los aspectos descritos en *S. cerevisiae* parecen estar presentes en *Arabidopsis*. Además del dominio quinasa, la proteína AtGCN2 incluye los dominios conservados que interactúan con la parte N-terminal de GCN1 y los dominios relacionados con histidil-tRNA sintetasa, y se ha demostrado que interactúa con los tRNA no cargados y presenta actividad en las isoformas de eIF2α en *Arabidopsis* (Li et al., 2013). Por otro lado, el gen de *Arabidopsis* complementa al mutante *gcn2* de levadura (Zhang et al., 2003) y una línea mutante *gcn2* de *Arabidopsis* no puede fosforilar a eIF2α (Zhang et al., 2008). Sin embargo, aunque parece claro que AtGCN2 fosforila a eIF2α bajo muchos estreses diferentes, si este proceso activa la detención de la traducción de manera similar en mamíferos y levaduras es discutible y la falta de comprensión total del sistema persiste.

Esta total similitud entre los sistemas GCN2 en diferentes eucariotas se extiende, como se ha comentado anteriormente, al gen *GCN4* en levadura, el cual codifica un factor de transcripción que se traduce durante la parada general de la síntesis de proteínas tras la fosforilación de eIF2α después de situaciones de estrés, y que posteriormente activará una batería de genes involucrados en la recuperación de estrés (Hinnebusch et al., 2005). No existe homólogo de GCN4 en plantas hasta la fecha, aunque el sistema de activación de este factor mediante pautas de lectura cortas en la zona reguladora podría estar funcionando en plantas (Von Arnimet al.; 2014). Del mismo modo, las proteínas IMPACT de humanos, cuyo ortólogo en levadura es Y1H1, y las cuales presentan unos dominios de interacción muy parecidos a los de GCN2, no presentan homólogos en plantas

(Faus et al., resultados no publicados). Estas proteínas IMPACT/ Y1H1 se unen a la proteína GCN1, evitando así la unión GCN1-GCN2 y la estimulación de eIF2α durante el ayuno de aminoácidos. La actividad de GCN2 también se ve reforzada por otros estreses como la inhibición del proteosoma, la radiación UV o la falta de glucosa. Castilho y sus colaboradores (2014) demostraron que IMPACT afecta directa y específicamente la activación de GCN2 bajo estas condiciones de estrés en mamíferos. Mostraron que la activación de GCN2 en mamíferos requiere de la unión con GCN1 y que IMPACT promueve la disolución del complejo GCN2-GCN1, ocurrió de forma similar en levaduras con el Y1H1. No parece que este sistema de regulación esté funcionando en plantas del mismo modo.

Otro de los aspectos que permaneció indeterminado fue precisamente la existencia de una proteína GCN1 en plantas y su papel en la activación de GCN2 junto con la fosforilación de eIF2α. En levaduras, GCN1 es absolutamente necesario para que GCN2 detecte los tRNA no cargados y, como resultado, el mutante *gnc1* knock-out es incapaz de activar a GCN2 ni de fosforilar a eIF2α en presencia de ayuno de aminoácidos (Marton et al., 1993). ILITHYA (ILA) es la única proteína en *Arabidopsis* que comparte homología con el gen *ScGCN1* y fue relacionada con la inmunidad de las plantas (Monaghan y Li et al., 2010). Los resultados recientes de Wang et al y los nuestros, muestran una supuesta interacción entre los dominios de unión de AtGCN2 e ILA, además de que los mutantes *ila* no pueden fosforilar a eIF2α reforzando la idea de que existe una vía funcional GCN en *Arabidopsis*. La presencia de un dominio similar a eFE3 en la parte media de la proteína ILA también sugiere su unión al ribosoma, donde se sabe que GCN1 promueve la función de GCN2 (Marton et al., 1997). Sin embargo, como se ha visto en este trabajo y se discutirá más adelante, los fenotipos de los mutantes de pérdida de función de ambos genes (GCN2 y GCN1) en *Arabidopsis* son muy diferentes, indicando que GCN1 participa en procesos independientes de GCN2, algo que no había sido apuntado hasta la fecha en ningún organismo.

Finalmente, el gen *SCORD5 (AtABCF3)* renombrado como *AtGCN20* por su homología con el gen de levadura, se aisló en un rastreo donde se buscaba rescatar bacterias virulentas mutantes deficientes en COR y se demostró que presentaba homología con *ScGNC20*, proponiendo que estuviera funcionalmente relacionado con ILITHYA (Zeng et al., 2011). GCN20 es un regulador positivo de GCN2 en levadura, y se cree que estimula la activación de GCN2 a través de los tRNA descargados (Garcia-Barrio et al., 2000). SCORD5, sin embargo, pertenece a una familia de transportadores de unión a ATP (ABC) y en *Arabi-*

dopsis existen cinco genes que presentan más del 90% de similitud de secuencia con GsGCN20 (Sanchez-Fernandez et al. ,2001, Faus et al., artículo en revisión). Aunque muchos de los fenotipos de los mutantes de pérdida de función de los genes ILA y estos transportadores coinciden, ninguna de las líneas mutantes de estos cinco genes es capaz de eliminar la fosforilación del factor eIF2α en condiciones de estrés, lo que pone en evidencia la implicación de los mismos en la regulación mediada por GCN2.

En resumen, el sistema centrado en GCN2 en plantas presenta elementos conservados con otros organismos eucariotas (la propia quinasa GCN2) pero otros no presentes (GCN4/ATF4, IMPACT/Y1H1) y otros con implicación con GCN2 pero aparentemente con otras funciones (ILA (GCN1), SCORD5(GCN20)), lo que implica que harán falta más estudios para dilucidar totalmente la relevancia de estas proteínas en el control de la expresión génica en plantas.

4.3. El sistema GCN2 se activa en plantas ante muchos estreses celulares, aunque no siempre va asociado a la parada de la traducción

Existen diferentes mecanismos reguladores que ayudan a la célula a adaptarse a distintas condiciones fisiológicas y de estrés ambiental, como el control traduccional y la traducción selectiva de mRNAs (Wek et al., 2006). La traducción de proteínas se divide en tres fases (iniciación, elongación y terminación,) y aunque todas ellas están sujetas a mecanismo de control, la iniciación es el paso regulado en la mayoría de los casos. Varios factores eucarióticos de iniciación de la traducción (eIFs) participan en dicha fase y la regulan, como por ejemplo los factores eIF2B y eIF2 (Kimball et al., 1998; Kleijn et al., 1998). La regulación de la traducción a estos niveles proporciona una rápida respuesta celular frente a cambios en las condiciones fisiológicas o situaciones de estrés.

La síntesis de proteínas se inhibe por las condiciones de estrés para evitar el mal plegamiento de las proteínas y proporcionar una correcta viabilidad celular. Esta respuesta forma parte de un mecanismo protector que se desencadena por distintos estímulos y se conoce como “respuesta integral o general al estrés”. La inhibición de la traducción se consigue mediante modificaciones postraduccionales de eIF2α (Sonenberg and Hinnebusch et al., 2007). La ruta canónica descrita para la activación de GCN2 en levaduras es en respuesta al ayuno de aminoácidos. Pero, como se ha visto, existen otros estreses que activan dicha ruta como por ejemplo el estrés producido por ayuno de purinas (Rolfes

and Hinnebusch et al., 1993), limitación de glucosa o crecimiento en fuente de carbono no fermentable como el etanol (Yang et al., 2000), alta salinidad (Goossens et al., 2001; Narasimhan et al., 2004), tratamiento con un agente alquilante como el metil metano sulfonato (MMS) (Natarajan et al., 2001) y tratamiento con rapamicina, compuesto que inhibe a las quinasas TOR1P y TORP2 (Valenzuela et al., 2001; Cherkasova and Hinnebusch et al., 2003; Kubota et al., 2003).

Como ya hemos comentado, se ha visto que eIF2α se fosforila, en diferentes organismos, de modo dependiente de GCN2 ante estreses distintos al ayuno de aminoácidos. Nosotros, en *Arabidopsis*, hemos visto como las plantas tras ser tratadas con 1 mM de glifosato fosforilan eIF2α de manera dependiente de GCN2. Del mismo modo ocurre tras ser expuestas a radiación UV (Faus et al., 2015; Llabata et at., 2019), así como a ácido acético y alta salinidad (Faus et al., resultados no publicados). Algunos de estos estreses también fueron vistos por Lageix, junto con el ayuno de purinas, la exposición al frío, el tratamiento con distintos herbicidas, tratamiento con ácido salicílico, metil jasmonato y ACC (ácido 1-aminociclopropano-1-carboxilico) (Lageix et al., 2008). Por tanto, en plantas, esta quinasa no actúa únicamente como sensor de aminoácidos, sino que parece tener un papel más general en la homeostasis celular antes situaciones de estrés.

Este circuito regulador basado en el sistema GCN, donde eIF2α fosforilado facilita la adaptación al estrés mediante la inhibición de la síntesis de proteínas, se encuentra muy conservado en levaduras y mamíferos. En *Arabidopsis* se ha estudiado la adaptación al estrés y la regulación traslacional a través de mutantes de AtGCN1 y AtGCN2 (Izquierdo et al., 2018). Se ha visto que AtGCN1 y AtGCN2 median la formación de P-eIF2α y la adaptación de la célula al ayuno de aminoácidos. Sin embargo, la formación de P-eIF2α no está relacionada con la detección general de la síntesis de proteínas en todas las situaciones anteriores. Izquierdo et al (2018) vio como tras exponer las plantas a un estrés mitocondrial, la inhibición del crecimiento y la síntesis de proteínas no varió entre las plántulas silvestres y las mutantes en GCN2, aunque estas últimas no acumulan P-eIF2α, lo que llevo a pensar que GCN2 y P-eIF2α no tienen una función central en la adaptación de la planta al estrés de los orgánulos, y que la fosforilación de eIF2α no causa necesariamente una inhibición general de la traducción. Estos resultados mostrarían la menor relevancia funcional de GCN2 y P-eIF2α en plantas en relación con otros eucariotas, al menos en este contexto.

En la misma línea, es de destacar cómo la perdida de función de GCN1, proteína imprescindible para la fosforilación de eIF2α, no implica un cese absoluto de la traducción, ya que mutantes en este gen siguen acumulando proteínas de modo similar a una planta silvestre (Faus et al., 2020). Aunque en este caso, las plantas no fueron sometidas a ninguna condición de estrés, este resultado es relevante, ya que incluso en condiciones no estresantes existe una fosforilación basal de eIF2α, necesaria seguramente para una regulación fina de este proceso celular (Murguía and Serrano et al., 2012).

4.4. Fenotipos de líneas mutantes en los componentes del sistema GCN2

En la década de los 80, *Arabidopsis thaliana* surgió como el sistema modelo escogido para comprobar o refutar modelos teóricos en plantas y en el año 2000 se convirtió en la primera planta con el genoma completo secuenciado (AGI, 2000). La importancia de conocer su genoma radica en que podemos estudiar cuestiones fundamentales de estructura y funcionalidad comunes en eucariotas, como diferencias específicas de la biología de las plantas (Meinke et al., 1998). Otras características que convierten a *Arabidopsis thaliana* en un modelo ideal para el estudio de la biología y la genética en vegetales es que posee un pequeño tamaño (30-40 cm de altura), un ciclo de vida corto (2-3 meses), una elevada producción de semillas (>10.000 semillas/planta), además de un genoma pequeño (125Mb) y completamente secuenciado (Meinke et al., 1998; Koornneef et al., 2010). Tiene diversos ecotipos, es decir, colecciones de germoplasma autofecundado en una localidad y en un tiempo determinado, entre los cuales *Lansberg erecta* y *Columbia* son los aceptados como estándar en muchos estudios (Koorneef et al., 2010).

Por otro lado, las herramientas de biología molecular como la mutagénesis, etiquetado de genes, rastreo de mutantes, clonaje posicional o la transformación genética mediante *Agrobacterium tumefaciens*, puede llevarse a cabo en esta planta modelo. Dicho esto, y dada la versatilidad de esta planta para el análisis genético, existen diversas técnicas para determinar la función de genes que participan en un determinado proceso. Estas estrategias se podrían clasificar en: estrategias de genética directa, de genética reversas, o las “ómicas”.

En las estrategias de genética directa, se selecciona de una población el individuo que posee el genotipo deseado, para luego determinar el gen o ruta causante de dicho feno-

tipo. Se puede emplear la variación natural o la inducida por agentes mutagénicos, como por ejemplo el EMS (Etil Metano Sulfonato), exposición a luz ultravioleta, transposones o inserciones de T-DNA transferido del plásmido T por *Agrobacterium tumefaciens* (Ostergaard et al., 2004). Tras ello, se procedería al rastreo del fenotipo escogido y se realizarían análisis genéticos y funcionales para determinar el gen mutado responsable de la variación en el fenotipo observado (Glazerbrook et al., 1996; Hong y Vierling, 2000).

Por otro lado, las estrategias de genética reversa se basan en una hipótesis previa según la cual un determinado gen "candidato" puede tener una función relevante en un proceso de interés, como por ejemplo la tolerancia a estrés. Partiendo de ello, se obtienen mutantes de perdida de función o sobreexpresados y se estudia los fenotipos de las plantas transgénicas obtenidas, además de inferir en el proceso en el que están implicados (Apse et al., 1999; Meissner et al., 1999).

Por último, las estrategias "omicas", técnicas basadas en análisis transcriptómicos, proteómicos o metabolómicos, tratan de estudiar los procesos de forma global, analizando los mRNA de los genes expresados (transcriptómica), proteínas traducidas (proteómica) o metabolitos producidos (metabolómica) por la planta en unas condiciones concretas, como un estrés. Los análisis globales se suelen comprobar con abordajes complementarios, como técnicas de genética reversa (Hilson et al., 2004, Brown et al., 2005).

Para evaluar la función de un gen mediante el uso de mutantes knock-out, es importante comprobar el fenotipo de las líneas mutantes en diferentes ecotipos. Si se obtiene el mismo resultado o tendencia en más de un ecotipo, la relación entre el fenotipo y el gen mutado puede deducirse fácilmente. Sin embargo, en algunos casos, solo las mutaciones realizadas en un ecotipo más sensible revelan fenotipos sutiles que, de lo contrario, no se detectarían si se analizan en ecotipos más robustos. Hasta donde sabemos, y durante el inicio de la realización de esta tesis, la única línea mutante homocigota disponible de GCN2 en *Arabidopsis* (GT8359) estaba en el ecotipo *Landsberg* (Ler) (Zhang et al., 2008). Para validar y contrastar los resultados publicados y nuevos, se inició la generación de una nueva línea mutante *gcn2* inactivada en el ecotipo *Columbia* (Col-0). La mutación se generó mediante la inserción de T-DNA de *Agrobacterium tumefaciens* en el genoma. Cuando se inserta en regiones genéticas, este T-DNA interrumpe el gen endógeno, provocando una supuesta desactivación del gen. Estas líneas, catalogadas como SALK, contienen al menos un inserto de T-DNA en una región conocida del genoma. En este caso, la

línea SALK_032196 tiene dos inserciones de T-DNA conocidas, ambas en una región exónica de los genes *AT3G59410 (GCN2)*, nuestro gen de interés, y el gen *AT5G18610*. Finalmente obtuvimos un mutante *gcn2* homocigoto en fondo Col-0 sin la inserción en el gen *AT5G18610*. Esta nueva línea se llamó *gcn2-2*, y el mutante original en Landsberg pasó a llamarse *gcn2-1*.

A nivel fenotípico y en condiciones normales de crecimiento, los mutantes *gcn2-1* y *gcn2-2* son prácticamente indistinguibles de sus correspondientes ecotipos silvestres (Lageix et al., 2008; Zhang et al., 2008; Faus et al., 2015; Liu et al., 2019; Llabata et al., 2019; Lokdarshi et al., 2020). Recientemente Lokdarshi vio como tras 3 días de exposición a altas intensidades de luz continuadas, el crecimiento de las plántulas *gcn2-1* se ve retrasado en comparación con su tipo silvestre, concretamente se vio afectada la longitud de la raíz y su peso fresco total, mientras que las plántulas *gcn2-1* crecidas en condiciones regulares de día y noche fueron normales. Dos alelos independientes de *gcn2* en fondo Columbia (Col-0), siendo uno de ellos *gcn2-2* (Faus et al., 2018; Llabata et al. ,2019) también tenían raíces más cortas tras 3 días de exposición a altas intensidades de luz continuada en comparación con el tipo silvestre, y después de la recuperación en luz normal, *gcn2-2* mostró menor peso fresco en comparación con su tipo silvestre. Estos alelos Col-0 también tenían raíces más cortas que el tipo silvestre bajo intensidades de luz normal continua, lo que sugirió que el fenotipo *gcn2* depende del ecotipo. Dado que tres alelos de pérdida de función de *gcn2* presentaron fenotipos similares a plántulas complementadas, concluyeron que la quinasa GCN2 en plantas juega un papel fisiológico en la adaptación al exceso de luz (Lokdarshi et al., 2020).

Mientras que las plantas *gcn2* son indistinguibles de su tipo silvestre, los alelos *gcn1* presentan claros fenotipos relacionados con el estrés y el desarrollo (Monaghan et al., 2010; Faus et al., 2018). La diferencia de fenotipo entre las plantas mutantes *gcn2-2* e *ila3* sugiere que promover la fosforilación de eIF2α por GCN2 no es la única función de la proteína ILA. La existencia de un alelo más fuerte (*ila2*), que muestra un fenotipo más severo que *ila3*, sugiere que la función de ILA independiente de eIF2α podría estar mediada por la región central, donde se encuentra la inserción de T-DNA en dicho mutante (Monaghan y Li et al., 2010). La interrupción de este supuesto dominio de unión al ribosoma podría estar afectando las propiedades de unión al ribosoma de esta proteína o a la unión de otros efectores involucrados en funciones, relacionadas o no, con la traducción; independientemente de la fosforilación de eIF2α. De hecho, este dominio similar a

EF3 en GCN1 también constituye el dominio de unión para la parte N-terminal de GCN20 en levadura (Marton et al., 1997). Otro dominio que forma parte de la proteína GCN20 muestra una gran similitud con la parte C-terminal de EFE, que abarca dos casetes de unión a ATP (ABC), por lo que el complejo GCN1/GCN20 cumpliría con las propiedades de unión a los ribosomas características del efecto EFE en levadura. Por otro lado, una de las proteínas homólogas de GCN20 en *Arabidopsis* (SCORD5) también presenta un fenotipo clorótico en las hojas emergentes (Faus et al., articulo en revisión), y además tanto la proteína ILA como SCORD5 no pueden cerrar los estomas después de una infección bacteriana y son susceptibles al ataque de patógenos (Zeng et al., 2011), lo que sugiere un enlace funcional entre las dos proteínas. Los resultados obtenidos por Izquierdo et al., 2018 y por nosotros (Faus et al., 2018) respaldan la teoría de una respuesta coordinada de GCN1 y GCN20 en respuesta a estreses ambientales independientemente de la acción de GCN2; nosotros observamos un comportamiento similar de ambos mutantes después de tratamientos con paraquat, NaCl y ácido abscísico, además de presentar defectos similares en el desarrollo de los cloroplastos. La tolerancia al paraquat, un potente producto de superóxido, podría explicarse porque la categoría "respuesta al estrés oxidativo" destacó por un aumento de expresión sobre el mutante *gcn20/scord5*, como sucedió con *Ila3* (Faus et al., 2015). El hecho de que la pérdida de función de cualquiera de estas proteínas confiera tolerancia al paraquat pero sean susceptibles al NaCl es intrigante, ya que el estrés oxidativo es un importante componente involucrado en el daño inducido por la salinidad (Moradi e Ismail et al., 2007), y la acumulación de NaCl es seguido por el aumento de superóxido y peróxido de hidrógeno (Mishra et al., 2013). Teniendo en cuenta que ambos mutantes son sensibles a ABA, compuesto que imita el estrés osmótico, sugerimos que la toxicidad del componente osmótico de NaCl podría ser la causa de los fenotipos observados.

El fenotipo clorótico de los mutantes *ila* sugirió que estas proteínas son necesarias para el correcto desarrollo y función de los cloroplastos. Las mediciones fotosintéticas y el análisis de los cloroplastos indican que la organización de los tilacoides y la fotosíntesis se ven afectadas en las hojas más jóvenes de ambas líneas mutantes (Faus et al. ,2018; Faus et al., artículo en revisión). Sin embargo, la estructura de los cloroplastos se mantiene, y no se ven tan gravemente dañados en comparación con otros mutantes deficientes en la formación de tilacoides, como *AtTerC* o *Thf1*, donde la matriz de los tilacoides se encuentra completamente alterada (Kwon y Cho et al., 2008; Wang et al., 2004). En los mutantes *ila*, la función de los cloroplastos se recupera a medida que las hojas com-

pletan su desarollo. Otros trabajos relacionados con las mutaciones ligadas a los cloroplastos describen unos efectos drásticos en las primeras etapas de desarrollo de la hoja que se superan en las etapas posteriores de desarrollo (Jarvis et al., 1998; Wang et al., 2004). Una posible explicación es que la planta tenga un mecanismo en el que compense la reducción de componentes importantes para el desarrollo del cloroplasto. En el caso de *ILA*, parece plausible que dicha compensación sea producida por los efectos de la interrupción del gen ILA, y no por la sustitución de la función molecular de la proteína para la cual codifican. Los transcriptomas de *ila3* y *gcn20* apoyan estos resultados fenotípicos previamente publicados. Los defectos en la biogénesis de los cloroplastos (Faus et al., 2018; Faus et al., en revisión) se evidencian por la baja regulación de genes relacionados con la fotosíntesis y los defectos similares observados en los cloroplastos de *ila3* y *gcn20*. El aumento de la expresión de genes implicados en la eliminación de superóxido en los tejidos de *ila3* podría indicar un contexto de estrés oxidativo en estos mutantes. Los mutantes del gen de la ferredoxina-NADP(+)oxidorreductasa, también presentan un fenotipo altamente deficiente en cloroplastos, además induce sistemas de eliminación de ROS para proteger a los cloroplastos dañados (Lintala et al., 2007; Lintala et al., 2012). Este mecanismo podría estar ocurriendo de forma similar en las hojas de *ila3* y *gcn20* como respuesta común para proteger los tejidos defectuosos resultantes de la mutación.

Una serie de proteínas “heat-shock” (HSP) aparecieron sobreexpresadas en el mutante *ila3* (Faus et al., 2018); Faus et al., 2020), esto podría entenderse como una respuesta para salvaguardar los daños en los tejidos provocados por las mutaciones (Rajan y D´Silva etal., 2009). Se ha descubierto que las HSP median la importación de los cloroplastos y las mitocondrias, incluido la HSP70 citosólica que se encuentra inducida en mutantes *ila3* (Flores-Perez y Jarvis, 2013). De manera similar a los *ila3* y *gcn20*, los mutantes de la subunidad CLPR2 del complejo de proteasas Clp también presentan un cloroplasto retardado y un desarrollo de la planta con un fenotipo verde pálido. Este complejo de proteasas es parte de la red de proteínas de la homeostasis del cloroplasto, y los mutantes de CLPR2 también sobreexpresan una batería de proteínas involucradas en el plegamiento e importación, incluidas varias HSP cloroplásticas (Lintala et al., 2007). Es importante señalar que la expresión reducida de estos genes no se dio en los mutantes *gcn2*. Los transcritos de plántulas de tipo silvestre y mutantes *gcn2-1* no revelaron grandes diferencias en la expresión génica entre ambos genotipos (Faus et al., 2015). Por tanto, es-

tos patrones de expresión génica alterados son presumiblemente independientes de la fosforilación de eIF2α.

Los datos fenotípicos y moleculares confirman aún más la asociación funcional de GCN1 y GCN20 de manera independiente de GCN2 y refuerza la idea de una nueva función para estas dos proteínas. Los ensayos de los mutantes *gcn1* y *gcn20* tras una infección por Pst DC3000 (Izquierdo et al., 2018) respaldan el papel de GCN1 y GCN20 en la respuesta preinvasiva a la infección bacteriana, en la que estas proteínas podrían regular la traducción de proteínas específicas. Sin embargo, los perfiles transcriptómicos basales de ambos mutantes indican que este nivel de regulación también podría ser determinante para este fenotipo.

Al comienzo de esta tesis, identificamos a GCN2 como un componente molecular que fomenta la acción del glifosato en *Arabidopsis* (Faus et al., 2015). Como ya se ha comentado, GCN2 es una proteína quinasa conservada, responsable de la fosforilación del factor de inicio eIF2α después de una serie de situaciones de estrés. En *Arabidopsis* se ha descrito que los tratamientos con herbicidas, las heridas, los tratamientos de choque térmicos por frío, exposición a luz UV o inanición de purinas activan a AtGCN2 (Lageix et al., 2008), la única proteína capaz de fosforilar a eIF2α en plantas (Zhang et al., 2008). La fosforilación de eIF2α evita nuevos ciclos de traducción de proteínas, y se supone que esto ayuda a la célula a conservar los recursos metabólicos hasta que se haya superado el impacto biológico producido por el estrés (Baena-Gonzalez et al., 2010). En este modelo, la activación debería ser beneficiosa para la planta poder hacer frente al estrés. Sin embargo, hemos demostrado que la presencia de GCN2 está facilitando de alguna manera la acción del herbicida, y las respuestas celulares al glifosato no se activan o atenúan en una línea mutante que no es capaz de fosforilar a eIF2α (Faus et al., 2015). Sabemos que las plantas mutantes en *gcn2* en *Arabidopsis* también son resistentes al estrés por NaCl (Faus et al., resultados no publicados). Sin embargo, este no es la primera vez que muestra que la falta de GCN2 confiere una ventaja cuando se aplica un estrés. En levadura, ScGCN2 actúa también como un factor negativo, lo que confiere efectos tóxicos sobre el crecimiento bajo estrés por NaCl. De esta forma, una cepa inactiva de GCN2 es capaz de crecer con normalidad por debajo de los 400mM de NaCl (Goossens et al., 2001). En células tumorales humanas, se demostró que HsGCN2 tiene un efecto proapoptótico inesperado bajo estrés por deficiencia de glucosa, y las células mutantes en *gcn2* pueden sobrevivir más que las de tipo silvestre en estas condiciones de estrés

(Muaddi et al., 2010). Finalmente, nosotros también hemos visto como las plantas mutantes en *gcn2* no son más sensibles, sino resistentes, a tratamientos con luz ultravioleta (Llabata et al., 2019). Como se ve, estos estreses y la ruta canónica de ayuno de aminoácidos pueden utilizar distintas vías que convergen en la fosforilación de eIF2α con resultados biológicos opuestos. Queda por saber si el mecanismo de resistencia es el mismo para todo tipo de estreses.

4.5. Resistencia al glifosato y UV-B de la línea mutante *gcn2*

El glifosato se ha convertido en el herbicida más utilizado en el mundo, especialmente tras la aparición de cultivos transgénicos resistentes en 1996. En el año 2007, más del 80% de los cultivos transgénicos del mundo, fueron diseñados para ser resistentes al glifosato (James C. et al., 2010). Sin embargo, el aumento de la frecuencia de aplicación del herbicida, ha producido una evolución de la resistencia al glifosato por parte de lo que se conocen como malas hierbas, provocando un problema que se ha convertido en una amenaza para la agricultura mundial. El glifosato afecta a las plantas sistemáticamente tras su aplicación por la superficie de las hojas. Los síntomas fitotóxicos se desarrollan lentamente, la muerte de la planta requiere días o semanas dependiendo de la dosis aplicada (Duke et al., 2008). La inhibición de su enzima principal, 5-enolpiruvilsikimato-3-fosfato sintasa (EPSPs; EC 2.5.1.19), inhibe la vía del ácido siquímico, lo que conduce a una escasez de aminoácidos aromáticos, quinonas y biosíntesis de cofactores. Algunos autores consideran esto como la principal causa de toxicidad del glifosato, además del lento desarrollo de los síntomas (Duke et al., 2008). Por el contrario, otros autores consideran que es debida a la acumulación del ácido siquímico (Fisher et al:, 1986; De Maria et al., 2006). Los herbicidas que inhiben la biosíntesis de aminoácidos también inducen efectos indirectos, como la proteólisis y un aumento de los aminoácidos libres (Zulet et al. ,2013).

Otro efecto del glifosato es la rápida represión de la fotosíntesis. La inhibición de la asimilación de CO_2 y el agotamiento de los intermediarios del ciclo de reducción del carbono se han estudiado con anterioridad (Geiger et al., 1986) y eso efectos se asociaron a un flujo regulado hacia arriba en la vía del ácido siquímico debido al agotamiento aguas abajo de EPSP. Ensayos transcriptómicos recientes revelaron que esta represión también podría estar genéticamente regulada. En un estudio comparativo con varias especies, se vió como tras 5 días de tratamiento con glifosato, se estaba produciendo una

marcada represión de los genes fotosintéticos, incluida la biosíntesis de clorofila, los fotosistemas y las enzimas del ciclo de Calvin (Cebeci et al , 2009). Nosotros también hemos observado una inducción en la expresión génica de los genes fotosintéticos en plantas adultas tras 15 días del tratamiento con glifosato, lo que encaja con una clara inhibición de la tasa fotosintética. La represión drástica de los genes fotosintéticos tras 6 horas del tratamiento de las plántulas de *Arabidopsis* con glifosato, cuando todavía no se observaban síntomas visibles de la clorosis en sus hojas, sugiere que además de una probable disminución fotosintética debido a la toxicidad metabólica, existe una inhibición temprana programada genéticamente de la fotosíntesis. Esta disminución de la fotosíntesis después del tratamiento con glifosato no se observa en las plantas mutantes *gcn2*, ni el estrés oxidativo característico del efecto del herbicida. Además, las enzimas que se sabe que están involucradas en la desintoxicación de herbicidas, como los transportadores ABC, las glutation-transferasas o las glucosiltransferasas (Edwards et al., 2005; Cummins et al., 2013) se activan drásticamente después del tratamiento con glifosato en las plantas de tipo silvestre, pero no se alteran o se activan de forma débil en las plantas mutantes *gcn2*. Por otro lado, la acumulación de ácido síquimico en las plantas *gcn2* en comparación con las plantas de tipo silvestre demuestra claramente que la falta de GCN2 se convierte en una ventaja cuando las plantas son tratadas con glifosato. El clorosulfuron, un tratamiento que bloquea la biosíntesis de valina, leucina y isoleucina (Zhang et al., 2008) produce un pico de fosforilación de eIF2α tras 6 horas del tratamiento, pero vuelve a los niveles basales pasadas 24 horas. La inanición de aminoácidos y la detención prolongada de la traducción de proteínas a través de GCN2 probablemente no sea la causa principal de los lentos efectos del tratamiento con glifosato. La participación de GCN2 en el modo de acción del glifosato debería caer en las primeras horas después del tratamiento, lo que condiciona el efecto final en la planta.

La activación temprana de GCN2 en plantas de tipo silvestre tras la aplicación de glifosato, seguramente debido a la descomposición inicial en aminoácidos aromáticos, no evita la expresión de factores celulares implicados en la desintoxicación de xenobióticos. En algunas especies el secuestro vacuolar contribuye al mecanismo de resistencia en variantes resistentes (Ge et al., 2014). Si este mecanismo ocurre en *Arabidopsis*, la activación de GCN2 podría afectar el tráfico de la membrana vacuolar a través de la inhibición de algunas proteínas importantes. Por otro lado, la traducción selectiva de ciertos mRNA, como ScGCN4 en levadura y HsATF4 en humanos (Hinnebusch et al., 2005) podría facilitar la translocación de glifosato a los tejidos jóvenes, donde se expresa

principalmente la enzima y la acción del glifosato es más visible (Duke et al., 2008). La reducción de la translación a los sumideros meristemáticos es un mecanismo importante de resistencias en algas (Feng et al., 2003), el Raigrás Italiano *(Lolium multiflorum)* (Perez-Jones et al., 2005) o *Conyza canadensis* (Koger et al., 2005). Sin embargo, hasta ahora no se ha encontrado una secuencia homologa a ScGCN4 en *Arabidopsis*, y se desconoce una traducción selectiva de mRNA dependiente de GCN2. La ausencia de actividad de GCN2 en la línea mutante *gcn2* podría proporcionar una ventaja constitutiva en el fondo mutante que disminuye los efectos de los herbicidas, independientemente de la activación de GCN2 tras el tratamiento. Otra posibilidad era una mayor absorción del glifosato por parte de las plantas de tipo silvestre. Aunque el mecanismo de absorción de glifosato en las células vegetales no se conoce bien, se ha propuesto la participación de transportadores de fosfato (Schmid et al., 1999; Morin et al., 1997). Las plantas *gcn2* no son más sensibles que las de tipo silvestre en ensayos de crecimiento con medios de cultivo con déficit de fósfato, y los experimentos de microarrays sobre plántulas no tratadas no revelaron diferencias en la expresión génica que haga sospechar de la falta de regulación del transporte de fosfato, lo que indica que el transporte de fosfato no está comprometido en las plantas *gcn2*. Si los transportadores de fosfato estuvieran involucrados en la absorción de glifosato en *Arabidopsis*, las plantas *gcn2* deberían tomar el herbicida a la misma velocidad que las plantas de tipo silvestre.

Por otro lado, la falta de GCN2 no confiere resistencia al glifosato en los ensayos de germinación. En un ensayo de plántulas realizado por Zhang et al., la línea mutante *gcn2* mostró sensibilidad al tratamiento con glifosato (Zhang et al., 2008). Sin embargo, nosotros no pudimos encontrar esta sensibilidad usando plántulas en la misma etapa de desarrollo en un experimento similar (datos no mostrados). La causa de estas discrepancias puede ser debida a diferencias sutiles en las condiciones experimentales. Se ha visto que un mutante *knock-out de gcn2* confiere resistencia y sensibilidad al mismo estrés en cultivos de células animales, GCN2 actúa como un interruptor molecular que desplaza las células de un estado proapoptótido a uno citoprotector en respuesta a la deficiencia de glucosa (Goossens et al., 2001) dependiendo de la duración del estrés.

En nuestra experiencia con el glifosato, hemos demostrado que el ácido siquímico no se acumula y que la activación de genes no se produce a la misma velocidad en el mutante *gcn2* que en las plantas de tipo silvestre, lo que indica que esta proteína quinasa podría ser una pista importante para descubrir componentes involucrados en la resistencia a

este herbicida. Dada la propagación de las malas hierbas resistentes al glifosato y la importancia económica para la agricultura, comprender estos mecanismos de resistencia podría ayudar a diseñar nuevas estrategias biotecnológicas para el uso más eficiente de esta importante herbicida.

Por otro lado, y como ya se ha comentado anteriormente a lo largo de este manuscrito, GCN2 es una proteína quinasa que regula la traducción de factores de transcripción como respuesta a diferentes estreses: ayuno de aminoácidos y purinas, shock por frío, heridas, cadmio y exposición a los rayos UV-C (Lageix et al., 2008). Estudios a nivel transcriptómico y proteómico han revelado que el exceso de exposición a luz UV-B interviene sobre los enlaces cruzados entre los RNA y proteínas del interior del ribosoma, además la recuperación celular viene acompañada de una mayor transcripción y traducción de genes relacionados con la síntesis de proteínas (Casati y Walbot et al., 2004).

Para investigar el papel de GCN2 en respuesta la radiación UV-B, se analizó su actividad a través de ensayos de fosforilación de eIF2α en mutantes de la vía de señalización específica del estrés de UV-B en *Arabidopsis*. En los experimentos realizados, la fosforilación de eIF2α por GCN2 fue detectable tras 30 minutos de exposición de UV-B, por lo tanto, su respuesta ante la inducción por UV-B es más rápida que la mayoría de las respuestas de expresión génica (Killian et al., 2007). La activación de GCN2 es independiente del fotorreceptor UV-B (UVR8) y de sus componentes de señalización aguas abajo y de la vía de señalización de estrés de las MAP quinasas MPK3, MPK6 o MKP1, lo que plantea la cuestión de en qué otras vías de señalización podría estar involucrada la proteína quinasa GCN2. En *Arabidopsis*, GCN2 es la única quinasa que fosforila a eIF2α bajo diferentes estreses, y como se muestras en esta tesis, también frente a la irradiación por UV-B.

Recientemente se ha demostrado que en mamíferos, GCN2 es más estimulado por los ribosomas que por los tRNA no cargados (Inglis et al., 2019). Por tanto, se podría especular que la exposición a UV-B induce cambios estructurales en el ribosoma que activan a GCN2 a nivel postranscripcional. En maíz se inducen proteínas ribosomales tras 2 horas de exposición a UV-B (Casati y Walbot et al., 2004), por lo que planteamos la hipótesis de que las proteínas ribosomales activan a GCN2 o son las responsables de la sobreacumulación de tRNA no cargados. GCN2 cuando es activado fosforila a eIF2α y por lo tanto cambia la población de mRNA traducidos. En mamíferos y levaduras, GCN2 inhibe la síntesis global de proteínas al tiempo que permite la traducción de mRNA seleccionados.

Estos mRNA contienen varios marcos de lectura abiertos dentro de la región 5´ no traducida (Sonenberg y Hinnebusch et al., 2009; Pakos-Zebruncka et al., 2016). Los estudios del proteoma en el maíz tras la irradiación por los UV-B han demostrado que los genes que codifican componentes de la maquinaria traduccional están sobreexpresados, lo que sugiere que los ribosomas se sintetizan nuevamente para reactivar la traducción (Casati y Walbot et al., 2003; Casati y Walbot et al., 2004).

Por todo ello, proponemos las siguientes explicaciones para los fenotipos observados en el mutante *gcn2* tras ser irradiado con UV-B. GCN2 se necesita en condiciones de estrés y participa en mantener el equilibrio energético de la planta. Regula la transcripción solo indirectamente, por ejemplo, apoyando la transcripción preferencial del marco de lectura abierto aguas arriba que contiene el mRNA. El hecho de que el gen CHS, relacionado con la luz UV-B se exprese más en condiciones de luz blanca indica que GCN2 podría tener también un impacto sobre la expresión génica en condiciones normales, sin estrés. GCN2 podría estar involucrado en la represión transcripcional de la energía necesaria para la biosíntesis de los metabolitos implicados en la protección del estrés, como los de la vía fenilpropanoide. En los mutantes *gcn2* tenemos la hipótesis de que estos metabolitos no se suprimirían y, por lo tanto, los mutantes *gcn2* contienen una protección constitutiva más alta contra el daño al DNA inducido por rayos de UV-B. Además la tasa de traducción es más alta en mutantes *gcn2* en comparación con el tipo silvestre y se encuentra menos afectada por la radiación de la luz UV-B.

Por ultimo, la mayor tolerancia en relación con los parámetros de crecimiento y la fecundidad de los mutantes *gcn2* a una dosis altas diarias de UV-B es probablemente el resultado de una acción combinada de los tres niveles de protección: transcripción constitutiva de un gen de biosíntesis para potenciar la defensa frente al UV-B, menor formación de CDP (dímeros de pirimidina) y mayores tasas de traducción bajo UV-B. El crecimiento de los mutantes *gcn2* en exposición continuada a UV-B respalda el papel de GCN2 como regulador negativo de las respuestas a la luz UV-B. La elevada resistencia de los mutantes *gcn2* a la exposición repetida a UV-B apunta a un papel crítico de GCN2 en la regulación de la traducción en UV-B (Llabata et al., 2019).

5. CONCLUSIONES

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Las conclusiones más generales obtenidas a partir de los resultados de la presente tesis doctoral son las siguientes. Además, en cada contexto biológico particular estudiado en cada artículo, podrían extraerse otras conclusiones más concretas.

1. La presencia de AtGCN2 (At3G59410) y ILITHYIA (At1G64790) son esenciales para la fosforilación de eIF2α en *Arabidopsis*. Aunque su genoma contiene 5 genes con secuencia similar al gen *GCN20* de levadura, (necesario para la correcta fosforilación de eIF2α en esta especie), todos ellos son dispensables en este proceso.
2. En *Arabidopsis*, GCN2 interacciona con el dominio C-terminal de ILITHYA, indicando que ambas proteínas participan en un mismo proceso molecular, previsiblemente la fosforilación de eIF2α.
3. Diferentes estreses abióticos, como la luz ultravioleta B o el ayuno de aminoácidos, activan la quinasa GCN2 y fosforilan eIF2α.
4. La activación de la quinasa GCN2 ante diferentes estreses no implica una mayor resistencia de las plantas a dichos estreses: plantas con pérdida de función en el gen *GCN2* son incapaces de fosforilar eIF2α, y sin embargo son más resistentes a estrés por luz ultravioleta o glifosato.
5. Plantas con pérdida de función en GCN2 presentan menos inhibición fotosintética y acumulan menos ácido siquímico que sus controles tras un tratamiento con glifosato. Plantas con pérdida de función en GCN2 presentan menos dímeros de pirimidina y menos inducción de defensas ante el estrés ultravioleta. Esto convierte a GCN2 en un componente que, de algún modo "entorpece" el disparo de las defensas frente a estos dos estreses.
6. La activación de GCN2 por estrés ultravioleta es independiente de las rutas de señalización conocidas a través de UVR8 y de las MAP quinasas MPK3, MPK6 o MKP1.
7. Plantas con pérdida de función en GCN1 son fenotípicamente muy diferentes a plantas con pérdida de función en GCN2: las primeras presentan defectos en la biogénesis de cloroplastos, el crecimiento radicular, así como múltiples alteraciones transcriptómicas y proteómicas. Las plantas con pérdida de función en GCN2 son indistinguibles de sus controles silvestres. Esto sugiere que, además de la ayuda en la fosforilación de eIF2α, la proteína GCN1 tiene un papel en *Arabidopsis* independiente de GCN2.

8. Plantas con pérdida de función en GCN1 presentan alteraciones en su respuesta a diversos estreses abióticos respecto a sus controles silvestre. Además, en muchos casos, ese comportamiento no es el mismo que presentan las plantas con pérdida de función en GCN2.
9. Plantas con pérdida de función en GCN20 (*abcf3*) presentan un fenotipo (defectos en cloroplastos, alteraciones transcriptómicas) y un comportamiento ante estreses abióticos similar a plantas con pérdida de función en GCN1, reforzando la idea de que ambas proteínas están implicadas en una función celular independiente de la activación de eIF2α vía GCN2.

6. BIBLIOGRAFÍA

Apse, M.P, Aharon, G.S, Snedden, W.A and Blumwald, E.: **Salt tolerance conferred by over expression of vacuolar Na+/H+ antiport in *Arabidopsis*.** *Science* 1999.Vol 285,no. 5431,p. 1256.

Baena-González, E., Rolland, F., Thevelein, J.M. and Sheen, J.: **A central integrator of transcription networks in plant stress and energy signaling.** *Nature* 2007. Vol 448, 23.

Baena-González, E. and Sheen, J.: **Convergent energy and stress signaling.** *Trends in Plant Science* 2008. Vol.13 No.9.

Baena-González E.: **Energy signaling in the regulation of gene expression during stress.** *Molecular Plant* 2010. 3(2):300–13.

Barbet, N. C., U. Schneider, S. B. Helliwell, I. Stansfield, M. F. Tuite, and M. N. Hall: **TOR controls translation initiation and early G1 progression in yeast.** *Molecular Biology of the Cell* 1996. v. 7, p. 25-42.

Berlanga JJ, Ventoso I, Harding HP, Deng J, Ron D, Sonenberg N, Carrasco L, de Haro C: **Antiviral effect of the mammalian translation initiation factor 2alpha kinase GCN2 against RNA viruses**. *European Molecular Biology Organisation Journal* 2006. 25:1730–1740.

Brown D.M., Zeef.A.H., Ellis J., Goodacre, R. and Turnera S.R.: **Identification of Novel Genes in *Arabidopsis* Involved in Secondary Cell Wall Formation Using Expression Profiling and Reverse Genetics.** *The Plant Cell* 2005. Vol. 17, 2281–2295

Bultynck, G., V. L. Heath, A. P. Majeed, J. M. Galan, R. Haguenauer-Tsapis, and M. S. Cyert: **Slm1 and slm2 are novel substrates of the calcineurin phosphatase required for heat stress-induced endocytosis of the yeast uracil permease.** *Molecular Biology of the Cell* 2006. v. 26, p. 4729-45.

Byrne E, Prosser I, Muttucumaru N, Curtis T, Wingler A, Powers S, Halford N: **Overexpression of GCN2-type protein kinase in wheat has profound effects on free amino acid concentration and gene expression.** *Plant Biotechnology Journal* 2012. 10(3):328-340

Casati, P., and Walbot, V.: **Gene expression profiling in response to ultraviolet radiation in maize genotypes with varying flavonoid content**. *Plant Physiology* 2003. 132, 1739–1754. doi: 10.1104/pp.103.022871

Casati, P., and Walbot, V.: **Crosslinking of ribosomal proteins to RNA in maize ribosomes by UV-B and its effects on translation.** *Plant Physiology* 2004. 136, 3319–3332. doi: 10.1104/pp.104.047043

Castilho B.A, Shanmugamb R, Silva R.C, Ramesh R, Himme B.M, Sattlegger E: **Keeping the eIF2 alpha kinase GCN2 in check.** *Molecular Cell Research* 2014.v 1843, 9, 1948-1968.

Cebeci O, Budak H. **Global Expression Patterns of Three Festuca Species Exposed to Different Doses of Glyphosate Using the Affymetrix GeneChip Wheat Genome Array**. *Comparative and Functional Genomics* 2009. Article ID 505701:10. doi: 10.1155/2009/505701.

Chen J.J: **Regulation of protein synthesis by the heme-regulated eIF2lpha kinase: relevance to anemias.** *Blood* 2007. 109(7):2693-2699

Cherkasova, V.A. and Hinnebusch, A.G.: **Translational control by TOR and TAP42 through dephosphorylation of eIF2's kinase GCN2.** *Genes & development* 2003. 17:859–872.

Clemens M.J: **PKR–a protein kinase regulated by doublestranded RNA.** *The International Journal of Biochemistry & Cell Biology* 1997. 29(7):945-949

Crespo, J. L., and M. N. Hall: **Elucidating TOR signaling and rapamycin action: lessons from Saccharomyces cerevisiae.** *Microbiology and Molecular Biology Reviews* 2002. v. 66, p. 579-91, table of contents.

Cummins I, Wortley DJ, Sabbadin F, He Z, Coxon CR, Straker HE, et al. **Key role for a glutathione transferase in multiple-herbicide resistance in grass weeds.** *Proceedings of the Natational Academy of Science of the U S A* 2013. 110(15):5812–7.

De Haro, C., Mendez, R., and Santoyo, J.: **The eIF-2alpha kinases and the control of protein synthesis.** *Federation of American Societies for Experimental Biology Journal* 1996.10, 1378-1387.

De María N, Becerril JM, García-Plazaola JI, Hernández A, De Felipe MR, Fernández-Pascual M.: **New insights on glyphosate mode of action in nodular metabolism: Role of shikimate accumulation.** *Journal of Agricultural and Food Chemistry* 2006. 54:2621–8.

Deng J, Harding HP, Raught B, Gingras AC, Berlanga JJ, Scheuner D, Kaufman RJ, Ron D, Sonenberg N: **Activation of GCN2 in UV irradiated cells inhibits translation.** *Current Biology* 2002. 12:1279–1286.

Dever, T.E., Feng, L., Wek, R.C., Cigan, A.M., Donahue, T.F., and Hinnebusch, A.G.:**Phosphorylation of initiation factor 2 alpha by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast.** *Cell* 1992. 68, 585-596.

Dever T.E, Chen J.J, Barber G.N, Cian A.M, Feng L, Donahue T.F, London I.M, Katze M.G, Hinnebusch A.G: **Mammalian eukaryotik initiation factor 2α kinases functionally substitute for GCN2 in the GCN4 translational control mechanisms of yeast.** *Procedures of National Academy of Science* 1993. 90: 4616-4620

Dever T.E, Yang W.M, Astrom S, Bystrom A.S, Hinnebusch A.G : **Modulation ot Trna(I) (Met), Eif2, and Eif-2b expression shows that Gcn4 translation is inversely coupled to the level of Eif-2-Center-Dot_Gtp-Center_Dot-Met-Trna(I)(Met) ternary complexes.** *Molecular and Cell Biology* 1995. 15:6351-6363

Deprost, D., Yao, l., Sormani, R., Moreau, M., Leterreux, G., Nicolai, M., Bedu, M., Robaglia, C. & Christian Meyer, C.: **The *Arabidopsis* TOR kinase links plant growth, yield, stress resistance and mRNA translation.** *Embo reports* 2007.Vol 8, No 9.

Donnelly N., Gorman A.M., Gupta S., Samali A.: **The eIF2α Kinase: Their Structures and Function.** *Cellular an Molecular Life Sciences* 2013. 70(19):3493-511.

Duke SO, Powles SB. **Glyphosate: a once-in-a-century herbicide.** *Pest Management Science* 2008. 64(4):319–25.

Edwards R, Del Buono D, Fordham M, Skipsey M, Brazier M, Dixon DP: **Differential induction of glutathione transferases and glucosyltransferases in wheat, maize and *Arabidopsis thaliana* by herbicide safeners.** *Zeitschrift Naturforsch for C.Journal of Biociences* 2005. 60(3–4):307–16.

Faus I, Zabalza A, Santiago J, Nebauer SG, Royuela M, Serrano R, Gadea J.: **Protein kinase GCN2 mediates responses to glyphosate in Arabidopsis.** *BMC Plant Biology* 2015. 15:14

Faus I, Niñoles R, Kesari V, Llabata P, Tam E, Nebauer SG, Santiago J, Hauser MT, Gadea J: **Arabidopsis ILITHYIA protein is necessary for proper chloroplast biogenesis and**

root devel- opment independent of eIF2α phosphorylation. *Journal of Plant Physiol*ogy 2018.224–225:173–182

Faus I., Niñoles R., Tárraga S., Gadea J.: **Proteomic Analysis of the *ila2* mutant of *Arabidopsis* links translational regulation with photosynthesis, protein folding and ribosomal proteins.** *Acta Physiologiae Plantarum* 2020. 42:57

Fisher RS, Berry A, Gaines CG, Jensen RA. **Comparative action of glyphosate as a trigger of energy drain in Eubacteria**. *Journal of Bacteriology* 1986.168:1147–54.

Flores-Perez, U., Jarvis, P.: **Molecular chaperone involvement in chloroplast protein import.** *Biochimimica et Biophysica acta (BBA)-Molecular Cell Research* 2013. Acta 1833 (2), 332–340.

Garcia-Barrio M, Dong J, Ufano S, Hinnebusch A.G: **Association of GCN1–GCN20 1733 regulatory complex with the N-terminus of eIF2alpha kinase GCN2 is required for GCN2 activation.** *European Molecular Biology Organisation Journal* 2000. 19: 1887–1899.

Ge X, Davignon DA, Ackerman JJ, Sammons D. **In Vivo 31P-NMR Studies of Glyphosate Uptake, Vacuolar Sequestration, and Tonoplast Pump Activity in Glyphosate Resistant Horseweed (Conyza canadensis).** *Plant Physiology* 2014. 166:1255–68.

Geiger DR, Kapitan SW, Tucci MA. **Glyphosate inhibits photosynthesis and allocation of carbon to starch in sugar beet leaves**. *Plant Physiology* 1986. 82:468–72.

Glazebrook, J., Rogers, E.E and Ausubel, F.M.: **Isolation of *Arabidopsis* Mutants With Enhanced Disease Susceptibility by Direct Screening.** *Genetics* 1996. 143 973-982.

Goossens A, Dever T.E, Pascual-Ahuir A, Serrano R: **The protein kinase Gcn2p mediates sodium toxicity in yeast.** *Journal of Biological Chemistry* 2001. 276(33):30753-30760.

Grallert B, Boye E: **The GCN2 kinase as a cell cycle regulator.** *Cell Cycle Goergetown Tex* 2007. 6: 2768-2772

Halford, N.G., Hey, s., Jhurreea, D., Laurie, S., Mckibbin, R.S., Zhang, Y. and paul, M.J.: **Highly conserved protein kinases involved in the regulation of carbon and amino acid metabolism.** *Journal of Experimental Botany* 2004. Vol. 55, No. 394, pp. 35±42

Halford, NG.:**Regulation of carbon and amino acid metabolism, roles of sucrose non-fermenting-1-related protein kinase-1 and general control nonderepressible-2-relat-**

ed protein kinase. *Advances in Botanical Research Including Advances in Plant Pathology* 2006. 43, 93–142.

Hall, M. N.: **The TOR signalling pathway and growth control in yeast.** *Biochemical Society Transactions* 1996. v. 24, p. 234-9.

Harding, H.P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D.: **Regulated translation initiation controls stress-induced gene expression in mammalian cells.** *Molecular Cell* 2000. 6, 1099-1108.

Harding HP, Calfon M, Urano F, Novoa I, Ron D.: **Transcriptional and translational control in the Mammalian unfolded protein response.** *Annual review of cell and developmental biology* 2002. 18:575-599

Harris T.E., Lawrence J.C: **Tor signaling.** *Science´s STKE* 2003. Vol.2003,Issue 212,pp.re 15.

Heitman, J., N. R. Movva, and M. N. Hall: **Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast**: *Science* 1991a. v. 253, p. 905-9.

Helliwell, S. B., P. Wagner, J. Kunz, M. Deuter-Reinhard, R. Henriquez, and M. N. Hall: **TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast.** *Molecular Biology of the Cell* 1994. v. 5, p. 105-18.

Helliwell, S. B., I. Howald, N. Barbet, and M. N. Hall: **TOR2 is part of two related signaling pathways coordinating cell growth in Saccharomyces cerevisiae.** *Genetics* 1998a. v. 148, p. 99-112.

Helliwell, S. B., A. Schmidt, Y. Ohya, and M. N. Hall: **The Rho1 effector Pkc1, but not Bni1, mediates signalling from Tor2 to the actin cytoskeleton.** *Current Biology* 1998b. v. 8, p. 1211-4.

Hey, S.J., Byrne, E. and Halford, N.G.: **The interface between metabolic and stress signalling.** *Annals of Botany* 2010. 105: 197–203.

Hinnebusch, A.G. and Natarajan, K.: **Gcn4p, a Master Regulator of Gene Expression, Is Controlled at Multiple Levels by Diverse Signals of Starvation and Stress**. *Eukaryotic cell* 2002. p. 22–32.

Hinnebusch A.G.: **Translational regulation of GCN4 and the general amino acid control of yeast.** *Annual Review of Microbiology* 2005. 59:407-450.

Holcik M., Sonenberg N.: **Translational control in stress and apoptosis**. *Nature Reviews Molecular Cell Biology* 2005. 6(4):318–327

Hong, S.W. and Vierling, E.: **Mutants of *Arabidopsis thaliana* defective in the acquisition of tolerance to high temperature stress.** *Proceedings of the Natational Academy of Science of the U S A* 2000. vol. 97, no. 8. 4392–4397

Immanuel T.M., Greenwood D.R., MacDiarmid R.M.: **A critical review of translation initiation factor eIF2α kinases in plants-regulating protein synthesis during stress.** *Fuctional Plant Biology* 2012. 39:717-735.

Inglis, A. J., Masson, G. R., Shao, S., Perisic, O., McLaughlin, S. H., Hegde, R. S.,: **Activation of GCN2 by the ribosomal P-stalk.** *Proceedings of the Natational Academy of Science of the U S A* 2019. 116, 4946–4954. doi: 10.1073/pnas.1813352116

Izquierdo Y, Kulasekaran S, Benito P, López B, Marcos R, Cascón T, Hamberg M, Castresana C.: ***Arabidopsis* nonresponding to oxylipins locus NOXY7 encodes a yeast GCN1 homolog that mediates noncanonical translation regulation and stress adaptation**. *Plant Cell & Environment* 2018. 41(6):1438–1452

James C. **Global status of Commercialized Biotech**/*GM Crops*. 2010. www.isaaa.org

Jarvis, P., Chen, L.J., Li, H., Peto, C.A., Fankhauser, C., Chory, J.,:**An *Arabidopsis* mutant defective in the plastid general protein import apparatus.** *Science 1*998. 282 (5386), 100–103.

Jenkins, G. M., A. Richards, T. Wahl, C. Mao, L. Obeid, and Y. Hannun: **Involvement of yeast sphingolipids in the heat stress response of Saccharomyces cerevisiae.** The Journal of Biologycal Chemistry 1997. v. 272, p. 32566-72.

John, F., Roffler, S., Wicker, T. and Ringli, C.: **Plant TOR signaling components.** *Plant Signaling & Behavior* 2011. 6:11, 1700-1705.

Johnston, A. J., Meier, P., Gheyselinck, J., Wuest, S. E., Federer, M., Schlagenhauf, E., ... Grossniklaus, U.: **Genetic subtraction profil- ing identifies genes essential for Arabidopsis reproduction and reveals interaction between the female gametophyte and the maternal sporophyte.** *Genome Biology* 2007. *8*, R204.

Kamada, Y., Y. Fujioka, N. N. Suzuki, F. Inagaki, S. Wullschleger, R. Loewith, M. N. Hall, and Y. Ohsumi: **Tor2 directly phosphorylates the AGC kinase Ypk2 to regulate actin polarization.** Molecular and Cellular Biology 2005. v. 25, p. 7239-48.

Kimball, S. R., Heinzinger, N. K., Horetsky, R. L. and Jefferson, L. S.: **Identification of Interprotein Interactions between the Subunits of Eukaryotic Initiation Factors eIF2 and eIF2B.** *Journal of Biological Chemistry* 1998. **273**(5): 3039-3044.

Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O.: **The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses**. *The Plant Journal* 2007.50, 347–363. doi: 10.1111/j.1365-313X.2007.03052.x

Kleijn, M., Scheper, G. C., Voorma, H. O. and Thomas, A. A. M.: **Regulation of translation initiation factors by signal transduction.** *European Journal of Biochemistry* 1998. **253**(3): 531- 544.

Koornneef, M. and Meinke, D.:**The development of *Arabidopsis* as a model plant**. *The Plant Journal* 2010. 61, 909–921.

Krohn M, Skjolberg H.C, Soltani H, Grallert B, and Boye E: **The G1-S checkpoint in fission yeast is not a general DNA damage checkpoint.** *Journal of Cell Science* 2008. 121: 4047–4054

Kubota, H., Obata, T., Ota, K., Sasaki, T. and Ito, T.: **Rapamycin-induced translational derepression of *GCN4* mRNA involves a novel mechanism for activation of the eIF2alpha kinase *GCN2***. *The Journal of Biology Chemistry* 2003. C300133200.

Kunz, J., R. Henriquez, U. Schneider, M. Deuter-Reinhard, N. R. Movva, and M. N. Hall:**Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression.** Cell 1993. v. 73, p. 585-96.

Kwon, K.C., Cho, M.H.:**Deletion of the chloroplast-localized AtTerC gene product in *Arabidopsis thaliana* leads to loss of the thylakoid membrane and to seedling lethality.** *The Plant Journal* 2008. 55 (3), 428–442.

Lageix, S., Lanet, E., Pouch-Pélissier, M.N., Espagnol, M.C., Robaglia, C., Deragon, J.M. and Pélissier, T.: **Arabidopsis eIF2α kinase GCN2 is essential for growth in stress conditions and is activated by wounding.** *BMC Plant Biology* 2008, 8:134.

Lee Y, Kim S.: **Dual role of methionyl-tRNA synthetase in the regulation of translation and tumor suppressor activity of aminoacyl-tRNA synthetase-interacting multifunctional protein-3.** *Proceedings of the Natatural Academy of Science of the USA* 2011. 108: 19635–19640.

Li M.W., AuYeung W.K., Lam H.M.: **The GCN2 homologue in *Arabidopsis* thaliana interacts with uncharged tRNA and uses *Arabidopsis* eIF2α molecules as direct substrates.** *Plant Biology* (Stuttg) 2013. 15(1):13–8.

Lindsley J.E., Rutter J.: **Nutrient sensing and metabolic decisions.** *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 2004. 139: 543–559.

Lintala, M., Allahverdiyeva, Y., Kidron, H., Piippo, M., Battchikova, N., Suorsa, M., Rintamaki, E., Salminen, T.A., Aro, E.-M., Mulo, P.: **Structural and functional characterization of ferredoxin-NADP(+)-oxidoreductase using knock-out mutants of *Arabidopsis*.** *The Plant Journal* 2007. 49 (6), 1041–1052.

Lintala, M., Lehtimaki, N., Benz, J.P., Jungfer, A., Soll, J., Aro, E.M., Bolter, B., Mulo, P.: **Depletion of leaf-type ferredoxin-NADP(+) oxidoreductase results in the per- manent induction of photoprotective mechanisms in Arabidopsis chloroplasts.** *The Plant Journal* 2012. 70 (5), 809–817.

Liu Y, Laszlo C, Liu W, Chen X, Evans SC, Wu S : **Regulation of G(1) arrest and apoptosis in hypoxia by PERK and GCN2- mediated eIF2alpha phosphorylation.** *Neoplasia* 2010. 12: 61–68.

Liu, X., Afrin, T., and Pajerowska-Mukhtar, K.M.: **Arabidopsis GCN2 kinase contributes to ABA homeostasis and stomatal immunity.** *Communications Biology* 2019. 2, 302.

Llabata P., Richter J., Faus I., Slominska-Durdasiak K., haber Zeh L., Gadea J., Hauser M.T.: **Involvement of the eIF2α Kinase GCN2 in UV-B Responses.** *Frontiers in Plant Science* 2019. 10:1492.

Loewith, R., E. Jacinto, S. Wullschleger, A. Lorberg, J. L. Crespo, D. Bonenfant, W. Oppliger, P. Jenoe, and M. N. Hall: **Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control.** Molecular Cell 2002. v. 10, p. 457-68.

Loewith, R. and Hall, M.N.: **Target of Rapamycin (TOR) in Nutrient Signaling and Growth Control.** *Genetics* 2011. Vol. 189, 1177–1201.

Lokdarshi A., Guan J., Urquidi Camacho R.A., Ki Cho S., Morgan P.W., Leonard M., Shimono M., Day B., Von Arnim A.G.: **Light Activates the Trasnlational Regulatory Kinase GCN2 via Reactive Oxygen Species Emanating from the Chloroplast.** *The Plan Cell* 2020. doi: 10.1105.

Lopez-Juez E., Jarvis R.P., Takeuchi A.,Page A.M., Chory J.: **New *Arabidopsis* Cue Mutants Suggest a Close Connection Between Plastid-And Phytochrome Regulation of Nuclear Gene Expression**. *Plant Physiology* 1998. 118(3):803-15

Lu W, Laszlo C.F, Miao Z, ChenH, Wu S: **The role of nitric-oxide synthase in the regulation of UVB light-induced phosphorylation of the alpha subunit of eukaryotic initiation factor 2.** *Journal of Biological Chemistry* 2009. 284: 24281–24288.

Lu W, Li L, Chen M, Zhou Z, Zhang W, Ping S :**Genome-wide transcriptional responses of Escherichia coli to glyphosate, a potent inhibitor of the shikimate pathway enzyme 5-enolpyruvylshikimate-3-phosphate synthase.** *Molecular Biosystems* 2013. 9(3):522–30.

Marton, M.J., Crouch, D., Hinnebusch, A.G.: **GCN1, a translational activator of GCN4 in Saccharomyces cerevisiae, is required for phosphorylation of eukaryotic translation initiation factor 2 by protein kinase GCN2.** *Molecular and Cellular Biology* 1993. Biol. 13 (June (6)), 3541–3556.

Marton, M.J., Vazquez De Aldana, C.R., Qiu, H., Chakraburtty, K. and Hinnebusch, A.G.: **Evidence that GCN1 and GCN20, Translational Regulators of GCN4, Function on Elongating Ribosomes in Activation of eIF2a Kinase GCN2.** *Molecular and cellular biology* 1997. p. 4474–4489.

Meinke, D.W., Cherry, J.M., Dean, C., Rounsley, S.D., Koornneef, M.: ***Arabidopsis thaliana*: A Model Plant for Genome Analysis**. *Science* 1998. VOL 282.

Meissnerm, R.C., Jin, H., Cominelli, E., Denekamp, M., Fuertes, A., Greco, R., Bevan, M.: **Function Search in a Large Transcription Factor Gene Family in Arabidopsis: Assessing the Potential of Reverse Genetics to Identify Insertional Mutations in R2R3 MYB Genes.** *The Plant Cell* 1999. Vol. 11, 1827–1840.

Mishra P, Bhoomika K, Dubey RS.: **Differential responses of antioxidative defense system to prolonged salinity stress in salt-tolerant and salt-sensitive Indica rice (Oryza sativa L.) seedlings.** *Protoplasma* 2013. Feb;250(1):3-19.

Monaghan, J. and Li, X.: **The HEAT Repeat Protein ILITYHIA is Required for Plant Immunity.** *Plant and Cell Physiology* 2002. 51(5): 742–753.

Monaghan, J., & Li, X.: **The HEAT repeat protein ILITYHIA is required for plant immunity.** *Plant and Cell Physiology* 2010. *51*, 742–753.

Moradi F, Ismail AM. (2007). **Responses of photosynthesis, chlorophyll fluorescence and ROS-scavenging systems to salt stress during seedling and reproductive stages in rice.** *Annals of Botany* 2007. Jun;99(6):1161-73.

Morin F, Vera V, Nurit F, Tissut M, Marigo G.: **Glyphosate uptake in Catharanthus roseus cells: Role of a phosphate transporter.** *Pesticide Biochemistry and Physiology* 1997. 58:13–22.

Muaddi H, Majumder M, Peidis P, Papadakis AI, Holcik M, Scheuner D, Kaufman RJ, Hatzoglou M Koromilas AE: **Phosphorylation of eIF2α at serine 51 is an important determinant of cell survival and adaptation to glucose deficiency.** *Molecular Biology Cell* 2010. 21(18):3220-31

Mulet, J. M., D. E. Martin, R. Loewith, and M. N. Hall: **Mutual antagonism of target of rapamycin and calcineurin signaling.** *Journal of Biological Chemistry* 2006. v. 281, p. 33000-7.

Muñoz A, Castellano M.M.: **Regulation of Translation Initiation under Abiotic Stress Conditions in Plants; Is It a Conserved or Not so Conserved Process among Eukaryotes?** *Comparative and Funtional Genomics* 2012. pp1-8.

Murguía J.R., Serrano R.: **New Functions of Protein Kinase Gcn2 in Yeast and Mammals.** *International Union of Biochemistry and Molecular Biology Life* 2012. 64(12): 971-974.

Narasimhan, J., Staschke, K. A. and Wek, R. C.: **Dimerization Is Required for Activation of eIF2 Kinase Gcn2 inResponse to Diverse Environmental Stress Conditions.** *Journal of Biological Chemistry* 2004. 279(22): 22820-22832.

Natarajan, K., Meyer, M.R, Jackson, B.M., Slade, D., Roberts, C., Hinnebusch, A.G. and Marton, M.J.: **Transcriptional Profiling Shows that Gcn4p Is a Master Regulator of Gene Expression during Amino Acid Starvation in Yeast.** *Molecular and cellular biology* 2001. p. 4347–4368.

Ostergaard, L. and Yanofsky, M.F.: **Establishing gene function by mutagenesis in *Arabidopsis thaliana*.** *The Plant Journal* 2004. 39, 682–696

Pakos-Zebrucka, K., Koryga, I., Mnich, K., Ljujic, M., Samali, A., and Gorman, A. M.: **The integrated stress response.** *The EMBO Journal* 2016. 17, 1374–1395. doi: 10.15252/embr.201642195

Rajan, V.B.V., D'Silva, P.,: ***Arabidopsis thaliana* J-class heat shock proteins: cellular stress sensors**. *Functional and Integrative Genomics* 2009. 9 (4), 433–446.

Rhoads R. E: **Regulation of eukaryotic protein synthesis by initiation factors**. *Journal of Biological Chemistry* 1993. 268: 3017–3020

Rolfes, R. J. and Hinnebusch, A. G.:**Translation of the yeast transcriptional activator *GCN4* is stimulated by purine limitation: implications for activation of the protein kinase GCN2.** *Molecular and cellular biology* 1993. **13**(8): 5099-111.

Rosa, M., Prado, C., Podazza, G., Interdonato, R., González, J.A., Hilal, M. and Prado, F.E.: **Soluble sugars—Metabolism, sensing and abiotic stress.** *Plant Signaling & Behavior* 2009. 4:5, 388-393.

Roy B, von Arnim A.G: **Translational Regulation of Cytoplasmic mRNAs**. *American Society of Plant Biologists* 2013. 1: 1-23.

Sánchez-Fernández R., Davies TG., Coleman JO.,Rea PA.:**The Arabidopsis thaliana ABC protein superfamily, a complete inventory.** *The Journal of Biological Chemistry* 2001. Aug 474 10;276(32):30231-44.

Sattlegger, E. and Hinnebusch, A.G.: **Separate Domains In GCN1 For Binding Protein Kinase GCN2 And Ribosomes Are Required For CGN2 Activation In Amino Acid-Starved Cells.** *The EMBO Journal* 2000. Vol. 19 No. 23. Pp 6622-6633.

Schmelzle, T. and Hall, M.N.: **TOR, a Central Controller of Cell Growth**. *Cell Review* 2000. Vol. 103, 253–262.

Schmid J, Amrhein N. T**he shikimate pathway. In: Singh B, editor. Plant Amino Acids, Biochemistry and Biotechnology.** *New York: Marcel Dekker* 1999. p. 147–70.

Segovia de Arana J.M, Albalat Criado C: **De Cajal a la ciencia actual**. *FarmaIndustria (serie científica)* 2007.

Sonenberg N, Hinnebusch A.G:**New Modes of Translational Control in Development, Behavior, and Disease.** *Molecular Cell* 2007. 136: 731–745

Sonenberg N, Hinnebusch A.G: **Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Targets.** *Cell* 2009. 136: 731–745

Tong-Tong Han, Wen-Cheng Liu, Ying-Tang Lu.:**General Control Non-Repressible 20 (GCN20) Functions in Root Growth by Modulating DNA Damage Repair in *Arabidopsis*.** *BMC Plant Biology* 2018.18(1):274.

Toroser, D., Plaut, Z. and Huber, S.C.: **Regulation of a Plant SNF1-Related Protein Kinase by Glucose-6-Phosphate.** *Plant Physiology* 2000. Vol. 123.

Tvegard T, Soltani H, Skjolberg H.C, Krohn M, Nilssen E.A, Kearsey S.E, Grallert B, and Boye E : **A novel checkpoint mechanism regulating the G1/S transition.** *Genes & Development* 2007. 21: 649–654

Valenzuela, L., Aranda, C. and González, A.: **TOR Modulates *GCN4*-Dependent Expression of Genes Turned on by Nitrogen Limitation.** *Journal of bacteriology* 2001. **183**(7): 2331- 2334.

Vattem, K.M. and Wek, R.C.: **Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells**. *Procedures of the National Academy of Science U.S.A* 2004. 101:11269–11274

Vazquez de Aldana C.R, Marton M.J, Hinnesbusch A.G.:**GCN20, a Novel ATP Binding Cassette Protein, and GCN1 Reside in a Complex That Mediates Activation of the eIF-2 Alpha Kinase GCN2 in Amino Acid-Starved Cells.** *The EMBO Journal* 1995. 3;14 (13): 3184-99.

Von Arnim A.G., Jia Q., Vaughn J.N. **Regulation of plant translation by upstream open reading frames.** *Plant Science* 2014. Jan;214:1-12.

Wang, Q., Sullivan, R.W., Kight, A., Henry, R.L., Huang, J., Jones, A.M., Korth, K.L.,:**Deletion of the chloroplast-localized Thylakoid formation1 gene product in *Arabidopsis***

leads to deficient thylakoid formation and variegated leaves. *Plant Physiology* 2004. 136 (3), 3594–3604.

Wang, L., Li, H., Zhao, C., Li, S., Kong, L., Wu, W., Kong, W., Liu, Y., Wei, Y., Zhu, J.K., Zhang, H.: **The inhibition of protein translation mediated by AtGCN1 is es- sential for cold tolerance in *Arabidopsis thaliana*.** *Plant Cell & Environment* 2016. 40 (1), 56–68.

Wek R.C, Jiang H.-Y, Anthony T.G: **Coping with stress:eIF2 kinases and translational control.** *Biochemical society* 2006. pp7-11.

Wilson, W.A. and Roach, P.J.: **Nutrient-Regulated Protein Minireview Kinases in Budding Yeast.** *Cell* 2002. Vol. 111, 155–158.

Wullschleger, S., R. Loewith, and M. N. Hall: **TOR signaling in growth and metabolism.** Cell 2006. v. 124, p. 471-84.

Xia X., Lei L., Qin W., Wang L., Zhang G., Hu J.: **GCN2 controls the cellular checkpoint: potential target fot regulating inflammation.** *Cell Death Discovery 2018.* 4:20

Xiong, Y. and Sheen, J.:**The Role of Target of Rapamycin Signaling Networks in Plant Growth and Metabolism.** *Plant Physiology* 2014. Vol. 164, pp. 499–512.

Yang R, Wek S.A, Wek R.C : **Glucose limitation induces GCN4 translation by activation of GCN2 protein kinase.** *Molecular Cell Biology* 2000. 20:2706–2717

Ye J, Kumanova M, Hart LS, Sloane K, Zhang H, De Panis DN, Bobrovnikova-Marjon E, Diehl JA, Ron D, Koumenis C.: **The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation.** *European Molecular Biology Organisation Journal* 2010. 29: 2082–2096

Zeng, W., Brutus, A., Kremer, J.M., Withers, J.C., Gao, X., Jones, A.D. and He, S.Y.: **A Genetic Screen Reveals *Arabidopsis* Stomatal and/or Apoplastic Defenses against Pseudomonas syringae pv. tomato DC3000.** *PLoS Pathogens* 2011. Volume 7, Issue 10, e1002291.

Zhang P, McGrath B.C, Reinert J, Olsen D.S, Lei L, Gill S, Wek S.A, Vattem K.M, Wek R.C, Kimball S.R, et al.: **The GCN2 eIF2αlphakinase is required for adaptation to amino acid deprivation in mice.** *Molecular and Cellular Biology* 2002. 22(19):6681-6688

Zhang Y, Dickinson J.R, Paul M.J, Halford N.G: **Molecular cloning of an *Arabidopsis* homologue of GCN2, a protein kinase involved in co-ordinated response to aminoacid starvation.** *Planta* 2003. 217: 668–675

Zhang Y, Wang Y, Kanyuka K, Parry MA, Powers SJ, Halford NG.: **GCN2-dependent phosphorylation of eukaryotic translation initiation factor- 2alpha in *Arabidopsis*.** *Journal of Experimental Botany* 2008. 59(11):3131–41.

Zhong L.J, Yang L, Lü F, Xiao H, Xu R, Wang L, Zhu F, Zhang Y : **UVA, UVB and UVC Induce Differential Response Signaling Pathways Converged on the eIF2α Phosphorylation.** *Photochemistry and Photobiology* 2011. 87:1092-1104.

Zhu J, Patzoldt WL, Shealy RT, Vodkin LO, Clough SJ, Tranel PJ.: T**ranscriptome response to glyphosate in sensitive and resistant soybean**. *Journal of Agricultural and Food Chemistry* 2008. 13;56(15):6355–63.

Zulet A, Gil-Monreal M, Villamor JG, Zabalza A, van der Hoorn RAL, Royuela M. **Proteolytic Pathways Induced by Herbicides That Inhibit Amino Acid Biosynthesis**. *PLoS One* 2013. 8(9):e73847.

7.ANEXO

frontiers in Plant Science

ORIGINAL RESEARCH
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Involvement of the eIF2α Kinase GCN2 in UV-B Responses

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Involvement of the eIF2a kinase GCN2 in UV-B responses

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Abstract

GCN2 (*GENERAL CONTROL NONREPRESSED2*) is a serine/threonine-protein kinase that regulates translation in response to stressors such as amino acid and purin deprivation, cold shock, wounding, cadmium and UV-C exposure. Activated *GCN2* phosphorylates the α-subunit of the eukaryotic translation initiation factor 2 (eIF2) leading to a drastic inhibition of protein synthesis and shifting translation to specific mRNAs. To investigate the role of *GCN2* in responses to UV-B radiation its activity was analyzed through eIF2α phosphorylation assays in mutants of the specific UV-B and stress signaling pathways of *Arabidopsis thaliana*. EIF2α phosphorylation was detectable 30 min after UV-B exposure, independent of the UV-B photoreceptor *UV RESISTANCE LOCUS8* and its downstream signaling components. GCN2 dependent phosphorylation of eIF2α was also detectable in mutants of the stress related MAP kinases, *MPK3* and *MPK6* and their negative regulator *MAP KINASE PHOSPHATASE1* (*MKP*1). Transcription of downstream components of the UV-B signaling pathway, the *CHALCONE SYNTHASE* (*CHS*) was constitutively higher in *gcn2-1* compared to wildtype and further increased upon UV-B while *GLUTATHIONE PEROXIDASE7* (*GPX7*) behaved similarly to wildtype. The UVR8 independent *FAD-LINKED OXIDOREDUCTASE* (*FADox*) had a lower basal expression in *gcn2-1* which was increased upon UV-B. Since high fluence rates of UV-B induce DNA damage the expression of the *RAS ASSOCIATED WITH DIABETES PROTEIN51* (*RAD51*) was quantified before and after UV-B. While the basal expression was similar to wildtype it was significantly less induced upon UV-B in the *gcn2-1* mutant. This expression pattern correlates with the finding that *gcn2* mutants develop less cyclobutane pyrimidine dimers (CPDs) after UV-B exposure. Quantification of translation with the puromycination assay revealed that *gcn2* mutants have an increased rate of translation which was also higher upon UV-B. Growth of *gcn2* mutants to chronic UV-B exposure support *GCN2*'s role as a negative regulator of UV-B responses. The elevated resistance of *gcn2* mutants towards repeated UV-B exposure points to a critical role of *GCN2* in the regulation of translation upon UV-B.

Introduction

UV-B (280-315 nm) is the most harmful radiation of the sun's spectrum reaching the biosphere. Thanks to the stratospheric ozone layer the extremely damaging solar UV-C (100-280 nm) is completely absorbed, while about 10% of the UV-B reaches the Earth's surface. Thus plants are naturally never exposed to UV-C but to high-energy UV-B wavelengths mainly above 295 nm. High levels of UV-B damages RNA, DNA and represses its

replication, impairs translation and proteins, triggers reactive oxygen species (ROS) and lead to severe growth retardation in maize and *Arabidopsis* (Jansen et al., 1998; Britt, 2004; Casati and Walbot, 2004a; Qüesta et al., 2013; Lario et al., 2015). However, low levels of UV-B serve as signal for development such as photomorphogenesis and inhibition of hypocotyl elongation. UV-B stimulates the synthesis of UV-B and ROS scavenging secondary metabolites of the phenylpropanoid pathway, for instance flavonoids and anthocyanins (Tilbrook et al., 2013; Jenkins, 2017; Liang et al., 2019). The nucleocytoplasmic UV RESISTANCE LOCUS8 (UVR8) is sensing UV-B (Rizzini et al., 2011; Christie et al., 2012; Wu et al., 2012). In the absence of UV-B UVR8 forms homodimers in the cytoplasm which dissociate upon photoreception. Monomeric UVR8 interacts with a key regulator of photomorphogenesis, the E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1). This interaction is essential for UVR8 accumulation in the nucleus (Oravecz et al., 2006; Favory et al., 2009). The UVR8/COP1 interaction is also crucial for the expression and stability of the transcription factors ELONGATED HYPOCOTYL5 (HY5) and its homolog HYH (Ulm et al., 2004; Stracke et al., 2010; Rizzini et al., 2011; Huang et al., 2013; Binkert et al., 2014). Brown and Jenkins (2008) found that UVR8 dependent and independent genes exhibit different needs for fluence rates. The UVR8-COP1-HY5/HYH specific pathway activates genes below 1 µmol m-2 s-1 or even lower (0.1 µmol m-2 s-1) while the independent genes were stimulated above 1 µmol m-2 s-1 UV-B. Among low fluence rate UVR8 dependent genes are HY5, HYH, and their downstream targets CHALCONE SYNTHASE (CHS) and GLUTATHIONE PEROXIDASE7 (GPX7). Among the UV-B induced but UVR8 independent genes are for example FAD-LINKED OXIDOREDUCTASE (FADox) (Brown and Jenkins, 2008).

High dose of UV-B activates also stress integrator genes such as the mitogen-activated protein kinases (MPKs), MPK3 and MPK6, and their negative regulator MPK PHOSPHATASE1 (MKP1). The functionality of these stress integrators has been shown by genetic analyses in *Arabidopsis* with *mpk3* and *mpk6* mutants that were more tolerant while *mkp1* mutants were hypersensitive to UV-B radiation (González Besteiro et al., 2011; González Besteiro and Ulm, 2013). Higher doses of UV-B trigger largely the formation of cyclobutane pyrimidine dimers (CPDs) and to approximately 25% of damaged bases, pyrimidine [6-4] pyrimidone dimers ([6-4] photoproducts; [6-4] PPs) (Britt et al., 1993; Britt, 2004). However, photolyases rapidly repair these pyrimidine dimers during photoreactivation which needs minimal amounts of visible or at least UV-A (315 nm – 400 nm)

or blue light. Higher doses of UV-B (4 μmol m^{-2} s^{-1}) also induce the expression of the recombinase *RAS ASSOCIATED WITH DIABETES PROTEIN51* (*RAD51*) (Ulm et al., 2004; Lang-Mladek et al., 2012). RAD51 is recruited to sites of double-strand DNA breaks (DSBs) but also to promoters of defense genes (Yan et al., 2013). RAD51 associates with proteins involved in the repair by homologous recombination (HR) (Chapman et al., 2012).

Studies on the transcriptomic and proteomic level revealed that excess UV-B mediate cross-links between RNA and proteins within the ribosomes and cellular recovery is accompanied with increased transcription and translation of genes involved in protein synthesis (Casati and Walbot, 2004a). These include ribosomal proteins, initiation and elongation factors, and ribosome recycling factors. Furthermore, rapid and transient phosphorylation of the 40S ribosomal protein S6 (RPS6) and its S6 kinase was detected within 15 min of UV-B exposure in maize (Casati and Walbot, 2004a). RPS6 is involved in the selective translation of specific mRNAs (preferentially ribosomal proteins and elongation factors). These mRNAs contain an oligopyrimidine stretch at the transcriptional start site (Meyuhas and Dreazen, 2009). Another group of proteins related to translation are members of the 80S ribosome, the RPL10 gene family. Quantification of protein synthesis upon UV-B exposure revealed that a heterozygous mutant of *Arabidopsis* rpl10A was hypersensitive to UV-B. While the rate of translation of wildtype and rpl10B and rpl10C mutants was reduced to 60% of control condition, it was even more affected in the heterozygous rpl10A after a 4 h exposure to UV-B (Ferreyra et al., 2010).

Apart of regulating translation at the ribosomal level, protein biosynthesis is controlled through a kinase phosphorylating the α-subunit of the Eukaryotic Initiation Factor2 (eIF2). EIF2α is required for the delivery of the initiator tRNAMet to the translation machinery. The evolutionary conserved protein kinase is GCN2 (general control nonrepressed2/ EIF2AK4). GCN2 plays a central role in modulating protein biosynthesis in response to different environmental stresses causing a nutritional imbalance. GCN2 strongly reduces global protein synthesis via phosphorylation of eIF2α from yeast to mammals. In plants, GCN2 is activated in response to amino acid starvation, stimulated by herbicides such as glyphosate and chlorsulfuron, by purine deprivation through guanine alkylation with methyl methanesulfonate, by exposure to UV-C and low temperature, by wounding and the stress hormones methyl jasmonate and salicylic acid along with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Lageix et al., 2008; Zhang et

al., 2008; Faus et al., 2018). Recently GCN2 has been assigned as carbon/nitrogen amino acid backbone sensor important for the biosynthesis of cysteine (Dong et al., 2017). Genetic analyses showed that GCN2 is the only kinase phosphorylating eIF2α under diverse stress conditions in the model plant *Arabidopsis thaliana* (Lageix et al., 2008; Zhang et al., 2008; Faus et al., 2018).

The aim of this study was to evaluate whether and how UV-B is activating GCN2 and which signaling pathway might be involved. Since GCN2 is a central regulator of translation the rate of translation in *gcn2* mutants in ambient and UV-B enriched light was quantified as well as CPD formation and repair. Growth characteristics revealed an increased tolerance of *gcn2* mutants towards chronic exposure to UV-B which correlated with a reduced CPD formation. The role of *GCN2* in UV-B triggered inhibition of translation is supported by *gcn2* mutants exhibiting a higher rate of translation upon UV-B compared to the wildtype backgrounds. The higher tolerance of *gcn2* mutants towards UV-B might in part be due to the constitutive higher expression of *CHS*, an early gene in the phenylpropanoid pathway and the increased ability to protect *gcn2* mutants from DNA damage.

1. Materials and Methods

1.1. Plant material and growth conditions

Arabidopsis thaliana Columbia accession (Col-0) and Landsberg *erecta* (Ler) were used as wild type controls. The T-DNA knock-out alleles in At5g18610, *gcn2-1* (GT8359) (Zhang et al., 2008) and *gcn2-2* which was purified from a second T-DNA insertion of SALKseq_032196 line (Faus et al., 2018) are in Ler and Col-0 background, respectively. The UV-B and stress signaling mutants *uvr8-6* (SALK_033468) (Favory et al., 2009), *cop1-4* (McNellis et al., 1994), *mpk6-2* (SALK_073907) (Nakagami et al., 2006) and *mpk3* (SALK_151594) (Nakagami et al., 2006) are in Col-0 background while *hy5-ks50* (Oyama et al., 1997), *hy5-ks50/hyh* (Holm et al., 2002), and *mkp1* (Ulm et al., 2001) are in Wassileskija (Ws).

1.2. Growth conditions, UV-C and UV-B treatments

Seeds were sterilized in 5% sodium hypochlorite as described by (Benfey et al., 1993). Sterile seeds were plated on MS (Duchefa Biochemie) medium supplemented with 4.5 % sucrose, 1% plant agar (Duchefa Biochemie). Seeds were stratified in the dark at 4°C for 48 h and transferred to a continuous light cabinet (RUMED, Rubath Apparate GmbH) with

22°C for 11-15 days. For experiments with soil grown plants, seedlings were transferred to soil (50 % potting soil, 50 % perlite) and cultivated at 20°C and 70% relative humidity (York, Austria) in a 16/8h light/dark cycle (Philips TLD36W/840) of 75 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR, 400–700 nm) until UV-B treatments or seed maturation. For UV-C treatments, 10 days old seedlings cultivated on solid MS plates were exposed for 20 min in a crosslinker (Hoefer, 254 nm, max. µJoules), recovered in liquid 1% MS medium supplemented with 1% sucrose for 1 h, snap frozen in liquid nitrogen and stored at –80°C. UV-B radiation started always 3 h after the onset of the day/night cycle (16 h light/8 h dark). For broad band UV-B exposure, approximately 25-30 days old soil grown plants were exposed for different times with 6 - 10 µmol m^{-2} s^{-1} (1.3-2.2 W/m^2) under Philips TL20W/12RS tubes in a growth chamber with 140–150 µmol m^{-2} s^{-1} of white light provided by Philips F17T8/TL741 fluorescent tubes (Philips, Amsterdam, Netherlands) and two additional HB GroLED lamps (CLF Plant Climatics, Wertingen, Germany). For chronic UV-B treatments, soil grown plants of about 25 days were exposed to 140–150 µmol m^{-2} s^{-1} of white light supplemented for 1 h/day with 6 µmol m^{-2} s^{-1} UV-B for 15 days. A cellulose di-acetate filter (ULTRAPHAN Acetatfolien®, 0.05 mm, Wettlinger Kunststoffe) was placed between the plants and the broad band UV-B lamps for filtered UV-B treatments. Philips narrowband TL20W/01-RS tubes were used with 3.5 µmol m^{-2} s^{-1} for narrow band UV-B treatments (spectra in **Supplementary Figure S1**). Fluence rates of white light (PAR) were measured using BlackComet C-UV/VIS spectrometer (StellarNet, Inc., Carlson, FL) and the SPECTRAWIZ® Software (Mainz, Germany). Fluence rates of UV-B (280–315 nm) were measured with a SKU435 UV-B sensor. The UV-B dosage was regulated by an Apogee UV-Sensor UVS (Model SU-100) positioned at the height of the rosette leaves about 40 cm below the UV-B tubes.

1.3. Phenotype evaluations

For the phenotypical evaluation rosette diameter, stem length and seed weight were quantified during and at the end of the chronic UV-B treatments. The rosette diameter was measured after 10 and 15 days at three positions of each rosette. Stem lengths were measured after stopping watering, when the plants were almost dry (around 12-15 days after the end of the UV-B treatment). The seeds were harvested from completely dry plants and weighed.

1.4. Western blots for GCN2 activity with Phospho-eIF2α (Ser51) specific antibody

Approximately 300 mg of leave tissue was ground with liquid nitrogen and resuspended with ice-cold 500 µL eIF2α extraction buffer (25 mM Tris-HCl pH 7.5, 75 mM NaCl, 5% glycerol, 0.05% Triton-X-100, 0.5 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 2 mM DTT, 2% PPV (polyvinyl pyrrolidone) containing protease (complete mini EDTA-free; Roche) and phosphatase inhibitors (20 mM ß-glycerolphosphat, 0.1 mM sodium orthovanadate (Na_3VO_4), 25 mM sodium fluoride). After centrifugation (Eppendorf centrifuge 5430R) for 30 min at 4ºC and 15000 g the supernatant was transferred to a new tube and centrifuged again for 15 min with the same settings. This supernatant was stored at -80ºC. After quantification with the Qubit Protein Assay Kit and the Qubit Fluorometer (both Invitrogen/Molecular Probes) 5x SDS-PAGE loading buffer was added to 20 µg protein and separated without prior heating on a 10% SDS-PAGE with 20 mA until the blue marker reached the end of the gel. After blotting the immunodetection was performed using 1:2000 diluted Phospho-eIF2α (Ser51) antibody (Cell Signaling Technology; #9721) and 1:10.000 diluted secondary ECL anti-rabbit IgG horseradish peroxidase antibody (GE Healthcare).

1.5. Quantification of the rate of global protein synthesis with puromycin specific antibody

The rate of global protein synthesis was quantified with puromycin (PU) labeled nascent proteins and detection of the incorporated PU by Western blots. Fifteen 10 days old seedlings were transferred into 6-well plates with sterile water and puromycin dihydrochloride (Carl Roth) was added to a final concentration of 65 µg mL^{-1} if not otherwise specified. After PU incubation for 2 h in the continuous light cabinet (RUMED, Rubath Apparate GmbH) seedlings were weighed and flash frozen in liquid nitrogen. Extraction buffer (25 mM Tris/HCl, pH7.5, 50 mM KCl, 5 mM MgCl2, 5 mM DTT, 0.5 mM PMSF) was added to pulverized plant material in a ratio 1:1 (µl:mg), vortexed thoroughly and solid residues were separated by centrifugation for 15 min at 13000 g and 4°C. Protein concentration of the supernatant was quantified with the Qubit system (Invitrogen). 15 µg of total protein were separated with 10% SDS-PAGEs. For Western blot, proteins were transferred onto PVDF membranes (Carl Roth). A second gel served as loading control and was stained over night with Coomassie Brilliant Blue (0.25% (w/v) in 45% ethanol/ 10% acetic acid). For dot blots, 1 µL of serial protein dilutions (1, 0.8, 0.64, 0.32, 0.16 µg $µL^{-1}$) were dropped on a dry nitrocellulose membrane (Roth, Germany) in triplicates. Membranes were first dried at room temperature for at least 20 min and subsequently baked at 80°C for 2 h pressed between two glass plates with filter papers in between.

Western and dot blot membranes were blocked with 5% milk powder in TBS-T. Incorporated PU was immunodetected with 1:10000 dilutions of mouse anti-puromycin antibodies for at least 3 h (MABE343 clone 12D10, Merck Millipore, Darmstadt, Germany) and 1:10000 diluted goat anti-mouse HRP-conjugated (New England Biolabs GmbH, NEB, Frankfurt am Main, Germany) secondary antibodies in TBS-T. Signal detection was done with the Roti-Lumin-Plus substrate (Carl Roth, Germany) and digitalized in the Fusion Pulse TS (Vilber, Germany). For normalization, Western blot membranes were washed after immunodetection again with TBS-T and total proteins were visualized with Ponceau S stain (0.5% (w/v) Ponceau S in 1% acetate; Carl Roth, Germany) and Coomassie Brilliant Blue (0.1% (w/v) in 40% ethanol/10% acetic acid). After de-staining with water or 40% ethanol/10% acetic acid, respectively, membranes were dried and the signal detection was performed with the ChemiDoc XRS+ (Bio-Rad). The rate of translation was determined by measuring the signal intensities of all lanes of Western blots and Coomassie stained gels as well as dots from dot blots with the EvolutionCapt software (Vilber, Germany) using rolling ball background subtraction. Signals of total proteins stained with Ponceau S or Coomassie Brilliant Blue on membranes were quantified with the Image Lab Software 5.1 (Bio-Rad) using local background subtraction. The volumes of the PU signal of each lane or dot were divided by the adjusted volumes of total protein signal and the input protein, for dot blots respectively. To compare experimental repetitions the data were normalized to the mean of the control conditions of each blot.

1.6. RNA isolation, cDNA synthesis and quantitative real-time PCR (RT-qPCR)

Total RNA isolation, cDNA synthesis and quantitative real-time PCR (RT-qPCR) were performed as previously described (Karsai et al., 2002; Lang-Mladek et al., 2012). Primer pairs used for amplification are listed in **Supplementary Table S1**. Quantitative real-time PCR was performed on a Rotor-Gene 3000 (Corbett, Qiagen, Hilden, Germany) in 14 µL reactions containing 5 pmol of each gene specific primer, 1 µL 1:10 diluted cDNA and the 5x HOT FIREPol EvaGreen® qPCR Mix Plus (Solis Biodyne, Tartu, Estonia). In total four different experiments (i.e. biological repeats) were quantified. Each cDNA was measured in triplicate. Amplification occurred after an initial denaturation (15 min/94 °C) in 40 cycles (94 °C/5 s – 54 °C/5 s – 66 °C/25 s + acquisition – 81 °C/15 s acquisition – 85 °C/15 s acquisition). To determine the PCR efficiencies of each run, a dilution series for each analyzed gene(s) was included. Melting curves were recorded between 65 °C and 99 °C at the end of each run. Gene expression was calculated using the efficiencies

of each gene with the RotorGene software (Version 6.0) and Excel (Pfaffl, 2001; Bustin et al., 2009). Relative expressions were normalized to the three reference genes the regulatory subunit of *PROTEIN PHOSPHATASE2* (*PP2A*), *TUBULIN BETA9* (*TUB9*) and *UBIQUITIN5* (*UBQ*) (for primer, fragment size and efficiencies see **Supplementary Table S1**).

1.7. DNA damage analyses

Plants were treated for 1 h with broad band UV-B and whole rosettes were harvested in 15 mL tubes immediately or after 4 h recovery and flash frozen. Genomic DNA was isolated with hexadecyltrimethylammonium bromide (CTAB) as follows: frozen material was ground in liquid N_2, suspended in 5 mL 2x CTAB buffer (2% (w/v) CTAB, 100 mM Tris/HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% (w/v) polyvinylpyrrolidone PVP40) and incubated for 20 min at 65°C. After cooling on ice, 5 mL chloroform was added and vortexed for 2 min. After centrifugation with 7500 g at 5 °C for 5 min the upper phase was transferred into a new tube and the DNA was precipitated with 4 mL isopropanol at room temperature. DNA was pelleted for 20 min at 5°C and 7500 g, washed with 500 µL 70% ethanol and dissolved in 100 µL 1x TE (pH 7.5) and 3 µL DNase free RNase A (Carl Roth, Germany). DNA was quantified using the Qubit system (Invitrogen) and solutions containing 396 ng DNA in 82.5 µL of 1x TE were prepared. DNA was denatured by adding 0.6 N NaOH to a final concentration of 0.3 N and incubated at room temperature for at least 10 min. A Roti®-Nylon plus (Carl Roth, Germany) membrane was soaked in water for 15 min. After assembling the Bio dot microfiltration apparatus (Bio-Rad) the membrane was washed with 200 µL dH_2O per well. The samples were applied in 25 µL aliquots with six technical repeats (60 ng), then washed twice with 200 µL of 0.4 N NaOH. The membrane was dried completely at room temperature and subsequently baked at 80°C for 2 h in-between filter papers and glass plates. For immundetection of the UV-B induced cyclobutane pyrimidine dimers (CPDs), the membrane was blocked for 1 h with 5% milk powder in TBS-T. CPDs were detected with 1:2000 mouse anti-CPD antibodies (Cosmo Bio Co., Ltd, Japan) over night at 5°C and 1:4000 goat anti-mouse-HRP (NEB) for 1 h as secondary antibody. Signal detection was done with the Roti®-Lumin plus substrate (Carl Roth, Germany) and digitalized with the ChemiDoc XRS+ (Bio-Rad). The signal intensities were quantified with the Image Lab Software 5.1 (Bio-Rad) using global background subtraction.

Statistical Analysis

The data was analyzed with Excel and is presented as means +/- standard error (SE). Statistical significance differences between the wildtype, *gcn* mutants in respect to phenotypes, DNA damage and rate of protein synthesis were determined by Student's t-test and $p < 0.05$ and marked with stars. One-Way analysis of variance (ANOVA) was used for the DNA damage analyses.

2. Results

2.1. Broad band UV-B radiation activates GCN2

In *Arabidopsis*, several studies confirmed GCN2 activation after exposure to UV-C, however little is known about the biologically more relevant UV-B radiation. To investigate the role of GCN2 in responses to UV-B its activity was analyzed through eIF2α phosphorylation assays in wildtype and *gcn2-1* mutants. First we determined whether eIF2α was detectably phosphorylated upon broad band UV-B in wildtype. Indeed eIF2α phosphorylation is evident after a 90 min exposure to 8 µmol m^{-2} s^{-1} and even 6 h after UV-B shut down. Since in *gcn2-1* mutants eIF2α phosphorylation under these conditions is absent GCN2 is the only kinase responsible (**Figure 1A**). We next determined whether cellulose diacetate filtered UV-B stimulates eIF2α phosphorylation and when eIF2α phosphorylation is detectable after the start of the UV-B exposure (**Figure 1B, C**). First signs of eIF2α phosphorylation were detectable already 30 min after the onset of broad band (**Figure 1B**) and filtered broad band UV-B (**Figure 1C**). However, eIF2α phosphorylation was neither detectable immediately after a 1.5 h UV-B exposure nor 2 h after shut down of the narrow band lamps with a wave length maximum of 311 nm (**Supplementary Figure S5).** These findings indicate that the activation of GCN2 lies between 290 nm and 308 nm.

2.2. GCN2 activation is independent of the UV-B photoreceptor and the stress signaling kinases MPK3 and MPK6

To determine whether the activation of GCN2 depends on the UVR8-COP1-HY5/HYH or a general, but different UV-B induced stress signaling pathway, a genetic approach was exploited. For the UV-B specific signaling pathway eIF2α phosphorylation was assessed in *uvr8-6* mutants and mutants of the key light regulator and UVR8 interaction partner *cop1-4* and the downstream transcription factors *hy5* and *hyh* (**Figure 2A,B**). In all these UV-B photoreceptor dependent mutants eIF2α phosphorylation was detected. Since the dose of UV-B sufficed to activate the UV-B stress response pathway eIF2α phosphoryla-

tion was also examined in mutants of the MAP kinases, *mpk3* and *mpk6* and their negative regulator *mkp1*. Independent if the broad band UV-B was filtered (data not shown) or not (**Figure 2B and Supplementary Figure S4B**) eIF2α phosphorylation was detectable in these stress signaling pathway mutants demonstrating that the activation of GCN2 is neither triggered by the UVR8-COP1-HY5/HYH nor the MAP kinases stress signaling pathway.

2.3. UV-B related genes are differentially expressed in *gcn2* mutants

UV-B is inducing the expression of specific genes which are indicative for different signaling pathways. Among them is the gene for the first enzyme in the flavonoid biosynthesis, the CHS synthase. CHS is induced in an UVR8-COP1-HY5/HYH dependent manner at low UV-B fluence rates but also at higher and stressful UV-B fluence rates (Brown and Jenkins, 2008; Lang-Mladek et al., 2012). *GPX7* also depends on the UVR8-COP1-HY5/HYH pathway and similarly to all glutathione peroxidases it is involved the protection against photooxidative stress (Chang et al., 2009). In contrast, FADox is UVR8-COP1-HY5/HYH independent and plays a role in the biosynthesis of 4-hydroxyindole-3-carbonyl nitrile (4-OH-ICN), a metabolite with cyanogenic function and important for pathogen defense (Brown and Jenkins, 2008; Rajniak et al., 2015). Since in our UV-B experiments unfiltered broad band with comparable to outdoor fluence rates was used the DNA damage responsive gene, RAD51, was included in the quantitative expression analyses. Surprisingly the CHS gene was constitutively higher expressed in gcn2-1 mutants compared to wildtype while the FADox expression was significantly lower and GPX7 and RAD51 had a similar expression levels to wildtype under control conditions (Figure 3A). CHS induction upon UV-B exposure was weaker and delayed in gcn2-1 compared wildtype (Figure 3B), while GPX7 behaved similar to wildtype (Figure 3C, D). Similar to the CHS gene also the induction of FADox expression was delayed in gcn2-1 mutants but reached 2 h after UV-B shut down a comparable level as wildtype. A rather unexpected surprise was the expression behavior of the DNA damage reporter gene RAD51. While in wildtype RAD51 was about 12 to 25 fold induced upon UV-B exposure this was not the case in gcn2-1 mutants (Figure 3E). RAD51 induction upon UV-B exposure was delayed in gcn2-1 compared to wildtype and never reached the expression level of wildtype (Figure 3E). These expression analyses indicate that the DNA damage signal is weaker in gcn2-1 mutants. One might speculate that the constitutive higher expression of a key gene in the phenylpropanoid biosynthesis pathway, CHS, is involved in the accumulation of UV-B scavenging

components, protecting gcn2-1 mutants from excess UV-B and therefore delaying and weakening the typical transcriptional responses to UV-B.

2.4. *Gcn2* mutants develop less CPDs upon UV-B but the repair is like in wildtype

Encouraged by the *RAD51* expression data, the level of UV-B induced CPDs were quantified in *gcn2* mutants and compared to wildtype with dot blot assays. For these analyses rosettes of soil grown plants of two *gcn2* alleles with different wildtype backgrounds (*gcn2-1* in Ler, *gcn2-2* in Col-0) were treated for 1 h with UV-B supplemented to white light and harvested immediately. To assess photorepair, a similar amount of rosette leaves were harvested 4 h after UV-B shut down. Overall Ler accumulated significantly less CPDs than Col-0 (**Figure 4A**). Furthermore, wildtype plants accumulated more CPDs compared to *gcn2* mutants (**Figure 4A**). The difference between CPDs immediately and 4 h after shut down of UV-B was used to calculate the recovery due photorepair (**Figure 4B,C**). Photorepair (recovery) was more effective in Col-0 than Ler (**Figure 4A,C**). The mutants exhibited no differences in their rate of photorepair compared to the respective wildtype backgrounds (**Figure 4C**). These results indicate that stress activated GCN2 inhibits properties that support DNA protection upon UV-B exposure while CPD repair through photolyases is not differentially regulated between wildtype and *gcn2* mutants.

2.5. The rate of translation is higher in *gcn2* mutants

Next we aimed to quantify the rate of translation of the gcn2 mutants in comparison to their wildtype backgrounds by employing both Western and dot blot analyses with the non-radioactive puromycination assay (Figure 5A-E). The puromycination or SUface SEnsing of Translation (SUnSET) method has been developed in mammalian cells and works also with plants (Schmidt et al., 2009; Van Hoewyk, 2016). A modified method called PUassociated nascent chain proteomics was used to directly monitor translation with a proteomic approach (Aviner et al.,2013; Aviner et al., 2014). In summary, multiple experiments have shown that the puromycination assay is a valid fast and cost-effective non-radioactive alternative to the classic 35S methionine/cysteine labeling methods for monitoring and quantifying the rate of global protein synthesis. As expected for the role as negative regulator of translation both gcn2 mutants had a significantly higher rate of protein synthesis (**Figure 5E**).

2.6. UV-B reduces the rate of translation to a lesser extent in *gcn2* mutants

It has been shown that the rate of polysomal loading and thus translation adapts to various environmental changes among them light (Juntawong and Bailey-Serres, 2012; Liu et al., 2012; Pal et al., 2013). To determine the effect on protein biosynthesis of unfiltered and filtered UV-B, rosette leaves of soil grown plants or seedlings were exposed together with PU for 1 h to UV-B and puromycylation was quantified at different time points (**Figure 6A**). Both UV-B treatments resulted in less PU incorporation into newly synthesized proteins compared to only white light controls. Protein synthesis decreased by about 20% and 60% after a 1 h exposure to filtered and unfiltered UV-B and a PU labeling period of 3 h, respectively (**Figure 6A**). Although Col-0 wildtype and the *gcn2-2* mutant seemed to maintain a higher rate of PU incorporation and thus translation as Ler and gcn2-1, this accession specific effect was not significant (Figure 6B). The p-values were between Col-0 and Ler under unfiltered UV-B p=0.2003, and under filtered UV-B p=0.2688, and between gcn2-2 and gcn2-1 under unfiltered UV-B p=0.6147 and under filtered UV-B p=0.785. The results are consistent with experiments of *Arabidopsis* leaves exposed for 4 h to filtered UV-B of similar intensity and quantification of the rate of translation through in v*ivo* [^{35}S]Met labeling (Ferreyra et al., 2010). The reduced rate of translation recovered rapidly (**Supplementary Figure S3**) indicating the fast and dynamic response of translation to changing UV-B and light conditions. Similar to the no UV-B control condition both *gcn2* alleles maintained a higher rate of translation upon filtered and unfiltered UV-B exposure.

2.7. *Gcn2* mutants are more tolerant to UV-B

Finally we examined the functional relevance of the transient misregulation of translation in *gcn2* mutants on growth parameters such as rosette size (diameter), stem length and fecundity quantified via the total seed weight (**Figure 7A-E**). Rosettes of both *gcn2* alleles developed larger under a daily exposure to filtered and unfiltered broad band UV-B compared to their respective wildtype accessions (**Figure 7C, E**). A similar difference was quantified with stem length, although only the Ler accession allele *gcn2-1* was significantly higher than wildtype (**Figure 7C**) probably because the Col-0 accession grew generally larger than Ler and did not reach their final height. Highly significant was the effect on total seed weight (**Figure 7D**). These results clearly demonstrate the importance of translational control. They also illustrate, that even transient misregulations, for example through a daily dose of 1 h elevated UV-B, generate dramatic effects on growth, the overall development and fecundity.

3. Discussion

Our GCN2 activation experiments show that eIF2α phosphorylation is detectable already after 30 min of UV-B between 290 nm and 308 nm and thus belongs to the early UV-B induced events being faster than most gene expression responses (Kilian et al., 2007). The GCN2 activation is independent of the UVR8-COP1-HY5/HYH and the MPK3, MPK6 and MKP1 stress signaling pathway. This poses the question which other signaling pathways might trigger GCN2. Recently it has been shown that UVR8 directly interacts apart from the E3 ubiquitin ligase COP1, with several transcription factors (Liang et al., 2019). For example the WRKY transcription factor, WRKY36, acts as a repressor upstream of HY5 (Yang et al., 2018) (**Figure 8**). WRKY36 is transcriptionally induced by UV-B in an UVR8 independent manner and its direct interaction with UVR8 depends on the presence of UVR8 in the same cellular compartment. Upon UV-B, UVR8 accumulates in the nucleus were it associates with WRKY36 and releases WRKY36 from the *HY5* promoter. The UVR8/WRKY36 interaction allows *HY5* transcription and consequently photomorphogenesis (Yang et al., 2018). It is important to note that WKRY36 interacts similar to COP1, with the C-terminus of UVR8 but not with COP1. Similar to *gcn2-1*, *CHS* is higher expressed in *wrky36* mutants already under white light conditions compared to wildtype. Yang et al. (2018) proposed that under white light conditions HY5 is not out-competed by WRKY36 on its own promoter resulting in a constitutive higher expression of *HY5* and consequently *CHS* (Yang et al., 2018). Nevertheless, UV-B specific *WRKY36* repression and *HY5* expression needs the presence of UVR8 and thus is different from the GCN2 pathway (**Figure 8**). There is still a need to clarify whether the presence of monomeric UVR8 in the nucleus is the only signal for the UV-B dependent transcriptionally upregulation of *WRKY36*. Another recently revealed novel UV-B pathway component was identified through the interaction of activated UVR8 with the brassinosteroid induced transcription factors BRI1-EMS-SUPPRESSOR1 (BES1/BRZ2) and its interaction partner BES1-INTERACTING MYC-LIKE1 (BIM1) (Liang et al., 2018). The interaction with UVR8 releases these two transcription factors from the promoters of brassinosteroid regulated genes and as a consequence growth and in particular cell expansion of the hypocotyls are inhibited (Liang et al., 2018). Since also this signaling pathway needs the presence of UVR8 it is unlikely to be involved in the activation of GCN2 upon UV-B (**Figure 8**).

Explanations are still elusive why different action spectra are necessary for UVR8 monomerization and UV-B induced *HY5* expression and whether UVR8 is the only UV-B photo-

receptor or if additional factors are needed to modulate the action of UVR8 (Brown et al., 2009; Díaz-Ramos et al., 2018). UV-B responsive phenomena in mutants of *uvr8* or due to mutant phenotypes and or photobiological studies indicate that UV-B signaling might also be triggered by other pathways (Ulm et al., 2004; Safrany et al., 2008; Gardner et al., 2009; Shinkle et al., 2010; Leasure et al., 2011; Lang-Mladek et al., 2012; Tilbrook et al., 2013; Xie et al., 2015; O'Hara et al., 2019). Studies also suggested that reduced pterin may be a chromophore for a putative UV-B photoreceptor (Galland and Senger, 1988; Takeda et al., 2014). In mammals two major UV-B pathways have been proposed (Fritsche et al., 2007). One pathway is initiated due to the formation of pyrimidine dimers. The other pathway is independent of DNA damage and involves the cell surface arylhydrocarbon receptor (AhR) (Fritsche et al., 2007; Esser et al., 2013; Pollet et al., 2018). Upon UV-B, tryptophan forms an AhR ligand which upon binding triggers the translocation of AhR to the nucleus where detoxification genes are induced (Fritsche et al., 2007). In addition, UVB-activated AhR initiates endocytosis of the Epidermal Growth Factor Receptor (EGFR) and activates EGFR dependent phosphorylation of the mammalian MAP kinases, ERK1/2 (Fritsche et al., 2007).

From yeast to mammals GCN2 belongs to the integrated stress response pathway which is critical for adaptation. This pathway promotes cellular recovery upon stresses by balancing nutrient availability with protein translation and growth. In *Arabidopsis*, GCN2 is the only kinase, which phosphorylates eIF2α upon several stress conditions and, as it is shown in this work, also UV-B. The molecular mechanism of GCN2 activation has been intensively studied in yeast and mammalian cells. Accordingly, GCN2 binds uncharged tRNAs leading to a conformational change which exposes its kinase domain. GCN2 interacts also with ribosomes and a regulatory complex of GCN1 and the ATP-binding cassette protein GCN20 (Sattlegger and Hinnebusch, 2000; Sattlegger and Hinnebusch, 2005; Castilho et al., 2014). Recently it has been shown that mammalian GCN2 is even higher stimulated by ribosomes and purified ribosomal P-stalk complexes than deacylated tRNAs (Inglis et al., 2019). Thus one might speculate that UV-B induces structural changes on the ribosome which activate GCN2 at a posttranscriptional level. RNA-ribosomal protein crosslinks can be induced in maize within 2 h of filtered broad band UV-B (Casati and Walbot, 2004a). Thus we hypothesize that these crosslinks induce structural changes that either activate GCN2 through direct binding, or is responsible for the overaccumulation of uncharged tRNAs. Activated GCN2 phosphorylates eIF2α and thereby changes the population of translated mRNAs. In yeast and mammalian cells GCN2 inhibits global

protein synthesis while allowing the translation of selected mRNAs. These mRNAs contain several short upstream ORFs (uORFs) in the 5' untranslated region (5' UTR) reviewed in (Sonenberg and Hinnebusch, 2009; Pakos-Zebrucka et al., 2016). Transcriptome and in particular proteome studies after UV-B exposure in maize have shown that genes coding for components of the translational machinery are overrepresented suggesting that ribosomes are newly synthesized for revival of translation (Casati and Walbot, 2003, 2004b).

We propose following explanation for the phenotypes of *gcn2* mutants upon UV-B radiation. GCN2 is needed under stressful conditions and involved in the balancing of energy use. GCN2 regulates transcription only indirectly by for example supporting the preferential transcription of uORF containing mRNAs. That the UV-B related *CHS* gene is higher expressed under control white light conditions indicate that GCN2 might have an impact on gene expression also under non-stressful conditions. GCN2 might be involved in the transcriptional repression of the energy demanding biosynthesis of stress protective metabolites such as components of the phenylpropanoid pathway. In *gcn2* mutants we hypothesize that these metabolites or other protective features would be not suppressed and thus *gcn2* mutants contain a constitutive higher protection against UV-B induced DNA damage. Therefore the DNA damage response gene *RAD51* is also less activated (**Figure 3E**) and CPD formation is reduced (**Figure 4**). Also the rate of translation is higher in *gcn2* mutants compared to wildtype and is less affected by UV-B (**Figure 6B**). The increased tolerance in relation to growth parameters and fecundity of *gcn2* mutants to a daily dose of elevated UV-B is probably the result of a combined action of all the three protection levels, i) constitutive transcription of a biosynthesis gene for potential UV-B scavenging components, ii) less CPD formation and iii) continuation of higher rates of translation under UV-B.

4. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5. Author Contributions

J.G. and M-T.H. designed and supervised the project. P.L. did together with IF the eIF2α phosphorylation assays. P.L. did the gene expression and together with M-T.H. the growth experiments. K.M.S.-D., L.Z. and J.R. performed the PU experiments and quanti-

fications. J.R. did the CPD quantifications. M.-T. H., J.G., J.R. and J.G. analyzed the data. P.L. and M.-T.H. wrote the draft. All authors discussed the results and commented on the manuscript.

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8. References

Aviner, R., Geiger, T., and Elroy-Stein, O. (2013). PUNCH-P for global translatome profiling. Translation **1,** e27516.

Aviner, R., Geiger, T., and Elroy-Stein, O. (2014). Genome-wide identification and quantification of protein synthesis in cultured cells and whole tissues by puromycin-associated nascent chain proteomics (PUNCH-P). Nat. Protocols **9,** 751-760.

Benfey, P.N., Linstead, P.J., Roberts, K., Schiefelbein, J.W., Hauser, M.T., and Aeschbacher, R.A. (1993). Root development in *Arabidopsis* - 4 mutants with dramatically altered root morphogenesis. Development **119,** 57-70.

Binkert, M., Kozma-Bognar, L., Terecskei, K., Veylder, L., Nagy, F., and Ulm, R. (2014). UV-B-responsive association of the *Arabidopsis* bZIP transcription factor ELONGATED HYPOCOTYL5 with target genes, including its own promoter. Plant Cell **26,** 4200-4213.

Britt, A., Chen, J., Wykoff, D., and Mitchell, D. (1993). A UV-sensitive mutant of *Arabidopsis* defective in the repair of pyrimidine-pyrimidinone(6-4) dimers. Science **261,** 1571-1574.

Britt, A.B. (2004). Repair of DNA damage induced by solar UV. Photosynthesis Research **81,** 105-112.

Brown, B.A., and Jenkins, G.I. (2008). UV-B Signaling Pathways with Different Fluence-Rate Response Profiles Are Distinguished in Mature *Arabidopsis* Leaf Tissue by Requirement for UVR8, HY5, and HYH. Plant Physiology **146,** 576-588.

Brown, B.A., Headland, L.R., and Jenkins, G.I. (2009). UV-B Action Spectrum for UVR8-Mediated HY5 Transcript Accumulation in Arabidopsis. Photochemistry and Photobiology **85,** 1147-1155.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., and Wittwer, C.T. (2009). The MIQE Guidelines: *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments. Clinical Chemistry **55,** 611-622.

Casati, P., and Walbot, V. (2003). Gene Expression Profiling in Response to Ultraviolet Radiation in Maize Genotypes with Varying Flavonoid Content. Plant Physiology **132,** 1739-1754.

Casati, P., and Walbot, V. (2004a). Crosslinking of Ribosomal Proteins to RNA in Maize Ribosomes by UV-B and Its Effects on Translation. Plant Physiology **136,** 3319-3332.

Casati, P., and Walbot, V. (2004b). Rapid transcriptome responses of maize (Zea mays) to UV-B in irradiated and shielded tissues. Genome Biology **5,** R16.

Castilho, B.A., Shanmugam, R., Silva, R.C., Ramesh, R., Himme, B.M., and Sattlegger, E. (2014). Keeping the eIF2 alpha kinase Gcn2 in check. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1843,** 1948-1968.

Chang, C.C.C., Ślesak, I., Jordá, L., Sotnikov, A., Melzer, M., Miszalski, Z., Mullineaux, P.M., Parker, J.E., Karpińska, B., and Karpiński, S. (2009). Arabidopsis Chloroplastic Glutathione Peroxidases Play a Role in Cross Talk between Photooxidative Stress and Immune Responses. Plant Physiology **150,** 670-683.

Chapman, J.R., Taylor, Martin R.G., and Boulton, Simon J. (2012). Playing the End Game: DNA Double-Strand Break Repair Pathway Choice. Molecular Cell **47,** 497-510.

Christie, J.M., Arvai, A.S., Baxter, K.J., Heilmann, M., Pratt, A.J., O'Hara, A., Kelly, S.M., Hothorn, M., Smith, B.O., Hitomi, K., Jenkins, G.I., and Getzoff, E.D. (2012). Plant UVR8 Photoreceptor Senses UV-B by Tryptophan-Mediated Disruption of Cross-Dimer Salt Bridges. Science **335,** 1492-1496.

Dong, Y., Silbermann, M., Speiser, A., Forieri, I., Linster, E., Poschet, G., Allboje Samami, A., Wanatabe, M., Sticht, C., Teleman, A.A., Deragon, J.-M., Saito, K., Hell, R., and Wirtz, M. (2017). Sulfur availability regulates plant growth via glucose-TOR signaling. Nature Communications **8,** 1174.

Díaz-Ramos, L.A., O'Hara, A., Kanagarajan, S., Farkas, D., Strid, Å., and Jenkins, G.I. (2018). Difference in the action spectra for UVR8 monomerisation and HY5 transcript accumulation in *Arabidopsis*. Photochemical & Photobiological Sciences **17,** 1108-1117.

Esser, C., Bargen, I., Weighardt, H., Haarmann-Stemmann, T., and Krutmann, J. (2013). Functions of the aryl hydrocarbon receptor in the skin. Seminars in Immunopathology **35,** 677-691.

Faus, I., Niñoles, R., Kesari, V., Llabata, P., Tam, E., Nebauer, S.G., Santiago, J., Hauser, M.T., and Gadea, J. (2018). *Arabidopsis* ILITHYIA protein is necessary for proper chloroplast biogenesis and root development independent of eIF2α phosphorylation. Journal of Plant Physiology **224-225,** 173-182.

Favory, J.-J., Stec, A., Gruber, H., Rizzini, L., Oravecz, A., Funk, M., Albert, A., Cloix, C., Jenkins, G.I., Oakeley, E.J., Seidlitz, H.K., Nagy, F., and Ulm, R. (2009). Interaction of COP1 and UVR8 regulates UV-B-induced photomorphogenesis and stress acclimation in Arabidopsis. EMBO J **28,** 591-601.

Ferreyra, M.L.F., Pezza, A., Biarc, J., Burlingame, A.L., and Casati, P. (2010). Plant L10 Ribosomal Proteins Have Different Roles during Development and Translation under Ultraviolet-B Stress. Plant Physiology **153,** 1878-1894.

Fritsche, E., Schäfer, C., Calles, C., Bernsmann, T., Bernshausen, T., Wurm, M., Hübenthal, U., Cline, J.E., Hajimiragha, H., Schroeder, P., Klotz, L.-O., Rannug, A., Fürst, P., Hanenberg, H., Abel, J., and Krutmann, J. (2007). Lightening up

the UV response by identification of the arylhydrocarbon receptor as a cytoplasmatic target for ultraviolet B radiation. Proceedings of the National Academy of Sciences **104,** 8851-8856.

Galland, P., and Senger, H. (1988). THE ROLE OF PTERINS IN THE PHOTORECEPTION AND METABOLISM OF PLANTS. Photochemistry and Photobiology **48,** 811-820.

Gardner, G., Lin, E.M., Loerer, H., and Brinkman, D. (2009). Photobiological properties of the inhibition of etiolated *Arabidopsis* seedling growth by ultraviolet-B irradiation. Plant, Cell & Environment **32,** 1573-1583.

González Besteiro, M.A., and Ulm, R. (2013). Phosphorylation and Stabilization of *Arabidopsis* MAP Kinase Phosphatase 1 in Response to UV-B Stress. Journal of Biological Chemistry **288,** 480-486.

González Besteiro, M.A., Bartels, S., Albert, A., and Ulm, R. (2011). Arabidopsis MAP kinase phosphatase 1 and its target MAP kinases 3 and 6 antagonistically determine UV-B stress tolerance, independent of the UVR8 photoreceptor pathway. The Plant Journal **68,** 727-737.

Holm, M., Ma, L.-G., Qu, L.-J., and Deng, X.-W. (2002). Two interacting bZIP proteins are direct targets of COP1-mediated control of light-dependent gene expression in Arabidopsis. Genes & Development **16,** 1247-1259.

Huang, X., Ouyang, X., Yang, P., Lau, O.S., Chen, L., Wei, N., and Deng, X.W. (2013). Conversion from CUL4-based COP1–SPA E3 apparatus to UVR8–COP1–SPA complexes underlies a distinct biochemical function of COP1 under UV-B. Proceedings of the National Academy of Sciences **110,** 16669-16674.

Inglis, A.J., Masson, G.R., Shao, S., Perisic, O., McLaughlin, S.H., Hegde, R.S., and Williams, R.L. (2019). Activation of GCN2 by the ribosomal P-stalk. Proceedings of the National Academy of Sciences **116,** 4946-4954.

Jansen, M.A.K., Gaba, V., and Greenberg, B.M. (1998). Higher plants and UV-B radiation: balancing damage, repair and acclimation. Trends in Plant Science **3,** 131-135.

Jenkins, G.I. (2017). Photomorphogenic Responses to Ultraviolet-B light. Plant, Cell & Environment, n/a-n/a.

Juntawong, P., and Bailey-Serres, J. (2012). Dynamic light regulation of translation status in *Arabidopsis thaliana*. Frontiers Plant Sci **3,** 66.

Karsai, A., Müller, S., Platz, S., and Hauser, M.T. (2002). Evaluation of a homemade SYBR green I reaction mixture for real-time PCR quantification of gene expression. Biotechniques **32,** 790-796.

Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., D'Angelo, C., Bornberg-Bauer, E., Kudla, J., and Harter, K. (2007). The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. The Plant Journal **50,** 347-363.

Lageix, S., Lanet, E., Pouch-Pelissier, M.-N., Espagnol, M.-C., Robaglia, C., Deragon, J.-M., and Pelissier, T. (2008). *Arabidopsis* eIF2alpha kinase GCN2 is essential for growth in stress conditions and is activated by wounding. BMC Plant Biology **8,** 134.

Lang-Mladek, C., Xie, L., Nigam, N., Chumak, N., Binkert, M., Neubert, S., and Hauser, M.-T. (2012). UV-B signaling pathways and fluence rate dependent transcriptional regulation of ARIADNE12. Physiologia Plantarum **145,** 527-539.

Lario, L.D., Botta, P., Casati, P., and Spampinato, C.P. (2015). Role of AtMSH7 in UV-B-induced DNA damage recognition and recombination. Journal of Experimental Botany **66,** 3019-3026.

Leasure, C.D., Tong, H.-Y., Hou, X.-W., Shelton, A., Minton, M., Esquerra, R., Roje, S., Hellmann, H., and He, Z.-H. (2011). root uv-b sensitive Mutants Are Suppressed by Specific Mutations in ASPARTATE AMINOTRANSFERASE2 and by Exogenous Vitamin B6. Molecular Plant **4,** 759-770.

Liang, T., Yang, Y., and Liu, H. (2019). Signal transduction mediated by the plant UV-B photoreceptor UVR8. New Phytologist **221,** 1247-1252.

Liang, T., Mei, S., Shi, C., Yang, Y., Peng, Y., Ma, L., Wang, F., Li, X., Huang, X., Yin, Y., and Liu, H. (2018). UVR8 Interacts with BES1 and BIM1 to Regulate Transcription and Photomorphogenesis in *Arabidopsis*. Developmental Cell **44,** 512-523.e515.

Liu, M.J., Wu, S.H., and Chen, H.M. (2012). Widespread translational control contributes to the regulation of Arabidopsis photomorphogenesis. Mol Syst Biol **8**.

McNellis, T.W., von Arnim, A.G., Araki, T., Komeda, Y., Miséra, S., and Deng, X.W. (1994). Genetic and molecular analysis of an allelic series of cop1 mutants sug-

gests functional roles for the multiple protein domains. The Plant Cell Online **6,** 487-500.

Meyuhas, O., and Dreazen, A. (2009). Chapter 3 Ribosomal Protein S6 Kinase: From TOP mRNAs to Cell Size. In Progress in Molecular Biology and Translational Science (Academic Press), pp. 109-153.

Nakagami, H., Soukupová, H., Schikora, A., Zárský, V., and Hirt, H. (2006). A Mitogen-activated Protein Kinase Kinase Kinase Mediates Reactive Oxygen Species Homeostasis in *Arabidopsis*. Journal of Biological Chemistry **281,** 38697-38704.

O'Hara, A., Headland, L.R., Díaz-Ramos, L.A., Morales, L.O., Strid, Å., and Jenkins, G.I. (2019). Regulation of *Arabidopsis* gene expression by low fluence rate UV-B independently of UVR8 and stress signaling. Photochemical & Photobiological Sciences **18,** 1675-1684.

Oravecz, A., Baumann, A., Máté, Z., Brzezinska, A., Molinier, J., Oakeley, E.J., Ádám, É., Schäfer, E., Nagy, F., and Ulm, R. (2006). CONSTITUTIVELY PHOTOMORPHOGENIC1 Is Required for the UV-B Response in Arabidopsis. The Plant Cell Online **18,** 1975-1990.

Oyama, T., Shimura, Y., and Okada, K. (1997). The Arabidopsis HY5 gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. Genes & Development **11,** 2983-2995.

Pakos-Zebrucka, K., Koryga, I., Mnich, K., Ljujic, M., Samali, A., and Gorman, A.M. (2016). The integrated stress response. EMBO reports **17,** 1374-1395.

Pal, S.K., Liput, M., Piques, M., Ishihara, H., Obata, T., Martins, M.C.M., Sulpice, R., van Dongen, J.T., Fernie, A.R., Yadav, U.P., Lunn, J.E., Usadel, B., and Stitt, M. (2013). Diurnal Changes of Polysome Loading Track Sucrose Content in the Rosette of Wild-Type Arabidopsis and the Starchless pgm Mutant. Plant Physiology **162,** 1246-1265.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res **29,** e45.

Pollet, M., Shaik, S., Mescher, M., Frauenstein, K., Tigges, J., Braun, S.A., Sondenheimer, K., Kaveh, M., Bruhs, A., Meller, S., Homey, B., Schwarz, A., Esser, C., Douki, T., Vogel, C.F.A., Krutmann, J., and Haarmann-Stemmann, T. (2018).

The AHR represses nucleotide excision repair and apoptosis and contributes to UV-induced skin carcinogenesis. Cell Death & Differentiation **25,** 1823-1836.

Qüesta, J., Fina, J., and Casati, P. (2013). DDM1 and ROS1 have a role in UV-B induced- and oxidative DNA damage in A. thaliana. Frontiers in Plant Science **4**.

Rajniak, J., Barco, B., Clay, N.K., and Sattely, E.S. (2015). A new cyanogenic metabolite in *Arabidopsis* required for inducible pathogen defence. Nature **525,** 376.

Rizzini, L., Favory, J.-J., Cloix, C., Faggionato, D., O'Hara, A., Kaiserli, E., Baumeister, R., Schäfer, E., Nagy, F., Jenkins, G.I., and Ulm, R. (2011). Perception of UV-B by the *Arabidopsis* UVR8 Protein. Science **332,** 103-106.

Safrany, J., Haasz, V., Mate, Z., Ciolfi, A., Feher, B., Oravecz, A., Stec, A., Dallmann, G., Morelli, G., Ulm, R., and Nagy, F. (2008). Identification of a novel cis-regulatory element for UV-B-induced transcription in Arabidopsis. The Plant Journal **54,** 402-414.

Sattlegger, E., and Hinnebusch, A.G. (2000). Separate domains in GCN1 for binding protein kinase GCN2 and ribosomes are required for GCN2 activation in amino acid-starved cells. EMBO Journal **19,** 6622-6633.

Sattlegger, E., and Hinnebusch, A.G. (2005). Polyribosome Binding by GCN1 Is Required for Full Activation of Eukaryotic Translation Initiation Factor 2α Kinase GCN2 during Amino Acid Starvation. Journal of Biological Chemistry **280,** 16514-16521.

Schmidt, E.K., Clavarino, G., Ceppi, M., and Pierre, P. (2009). SUnSET, a nonradioactive method to monitor protein synthesis. Nat Meth **6,** 275-277.

Shinkle, J.R., Edwards, M.C., Koenig, A., Shaltz, A., and Barnes, P.W. (2010). Photomorphogenic regulation of increases in UV-absorbing pigments in cucumber (Cucumis sativus) and *Arabidopsis thaliana* seedlings induced by different UV-B and UV-C wavebands. Physiologia Plantarum **138,** 113-121.

Sonenberg, N., and Hinnebusch, A.G. (2009). Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Targets. Cell **136,** 731-745.

Stracke, R., Favory, J.-J., Gruber, H., Bartelniewoehner, L., Bartels, S., Binkert, M., Funk, M., Weisshaar, B., and Ulm, R. (2010). The Arabidopsis bZIP transcription factor HY5 regulates expression of the PFG1/MYB12 gene in response to light and ultraviolet-B radiation. Plant, Cell & Environment **33,** 88-103.

Takeda, J., Nakata, R., Ueno, H., Murakami, A., Iseki, M., and Watanabe, M. (2014). Possible Involvement of a Tetrahydrobiopterin in Photoreception for UV-B-induced Anthocyanin Synthesis in Carrot. Photochemistry and Photobiology **90,** 1043-1049.

Tilbrook, K., Arongaus, A.B., Binkert, M., Heijde, M., Yin, R., and Ulm, R. (2013). The UVR8 UV-B Photoreceptor: Perception, Signaling and Response. The *Arabidopsis* Book, e0164.

Ulm, R., Revenkova, E., di Sansebastiano, G.-P., Bechtold, N., and Paszkowski, J. (2001). Mitogen-activated protein kinase phosphatase is required for genotoxic stress relief in *Arabidopsis*. Genes & Development **15,** 699-709.

Ulm, R., Baumann, A., Oravecz, A., Máté, Z., Ádám, É., Oakeley, E.J., Schäfer, E., and Nagy, F. (2004). Genome-wide analysis of gene expression reveals function of the bZIP transcription factor HY5 in the UV-B response of Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **101,** 1397-1402.

Van Hoewyk, D. (2016). Use of the non-radioactive SUnSET method to detect decreased protein synthesis in proteasome inhibited Arabidopsis roots. Plant Methods **12,** 20.

Wu, D., Hu, Q., Yan, Z., Chen, W., Yan, C., and Huang, X. (2012). Structural basis of ultraviolet-B perception by UVR8. Nature **484**.

Xie, L., Lang-Mladek, C., Richter, J., Nigam, N., and Hauser, M.T. (2015). UV-B induction of the E3 ligase ARIADNE12 depends on CONSTITUTIVELY PHOTOMORPHOGENIC 1. Plant Physiology and Biochemistry **93,** 18-28.

Yan, S., Wang, W., Marqués, J., Mohan, R., Saleh, A., Durrant, Wendy E., Song, J., and Dong, X. (2013). Salicylic Acid Activates DNA Damage Responses to Potentiate Plant Immunity. Molecular Cell **52,** 602-610.

Yang, Y., Liang, T., Zhang, L., Shao, K., Gu, X., Shang, R., Shi, N., Li, X., Zhang, P., and Liu, H. (2018). UVR8 interacts with WRKY36 to regulate HY5 transcription and hypocotyl elongation in Arabidopsis. Nature Plants **4,** 98-107.

Zhang, Y., Wang, Y., Kanyuka, K., Parry, M.A.J., Powers, S.J., and Halford, N.G. (2008). GCN2-dependent phosphorylation of eukaryotic translation initiation factor-2α in Arabidopsis. Journal of Experimental Botany **59,** 3131-3141.

9. Figure legends

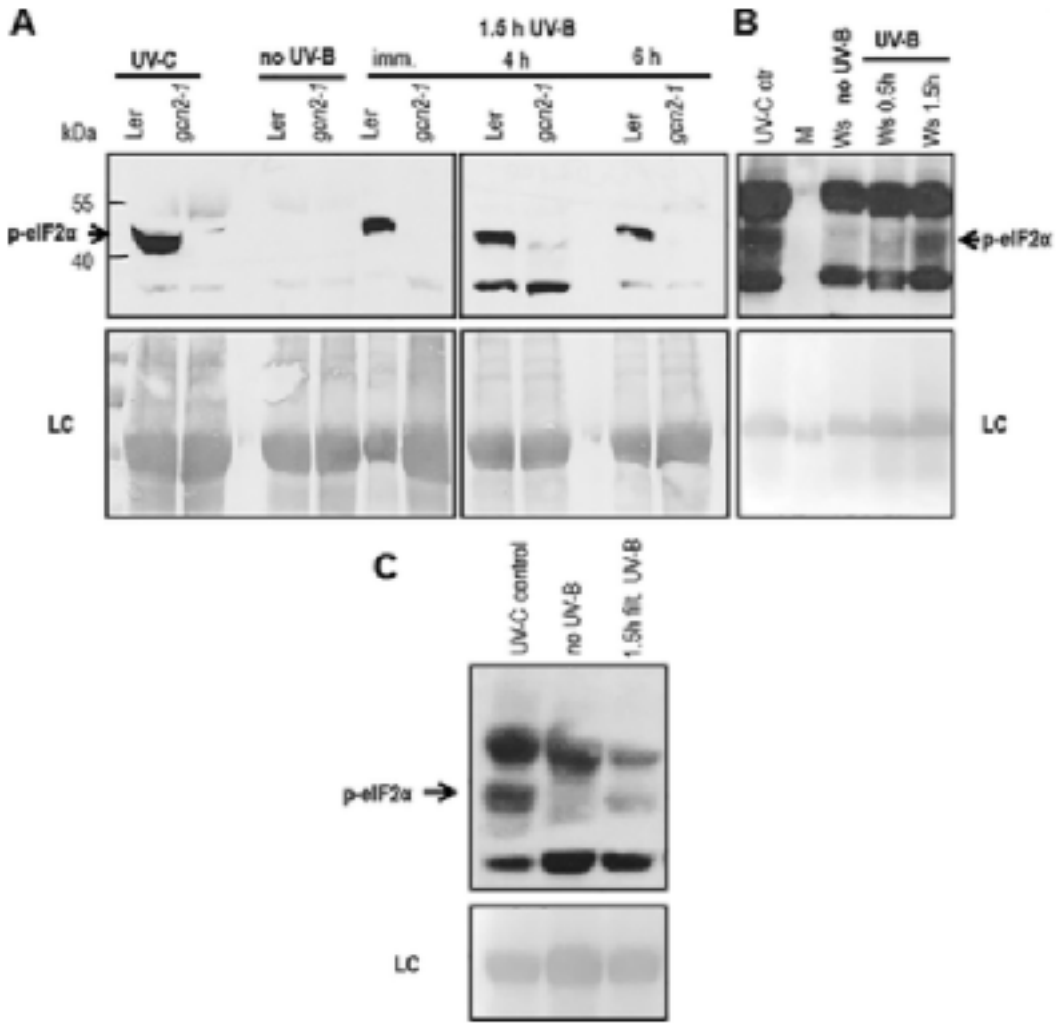

Figure 1. Western blots assessing the activation of GCN2 via eIF2α phosphorylation. (A) Wildtype (Ler) and gcn2-1 plants were either treated 20 min with UV-C (control) or with white light supplemented for 1.5 h with 8 µmol m-2 s-1 broad band UV-B. Leaves were harvested immediately (imm.), 4 h and 6 h after UV-B shut down. (B) First signs of eIF2α phosphorylation (p-eIF2α) is detectable already 30 min after the onset of 10 µmol m-2 s-1 broad UV-B. (C) Also 1.5 h of filtered broad band UV-B exposure is activating GCN2. Equal amount of protein (20 µg) was loaded on 10% SDS-PAGEs. LC: Loading control.

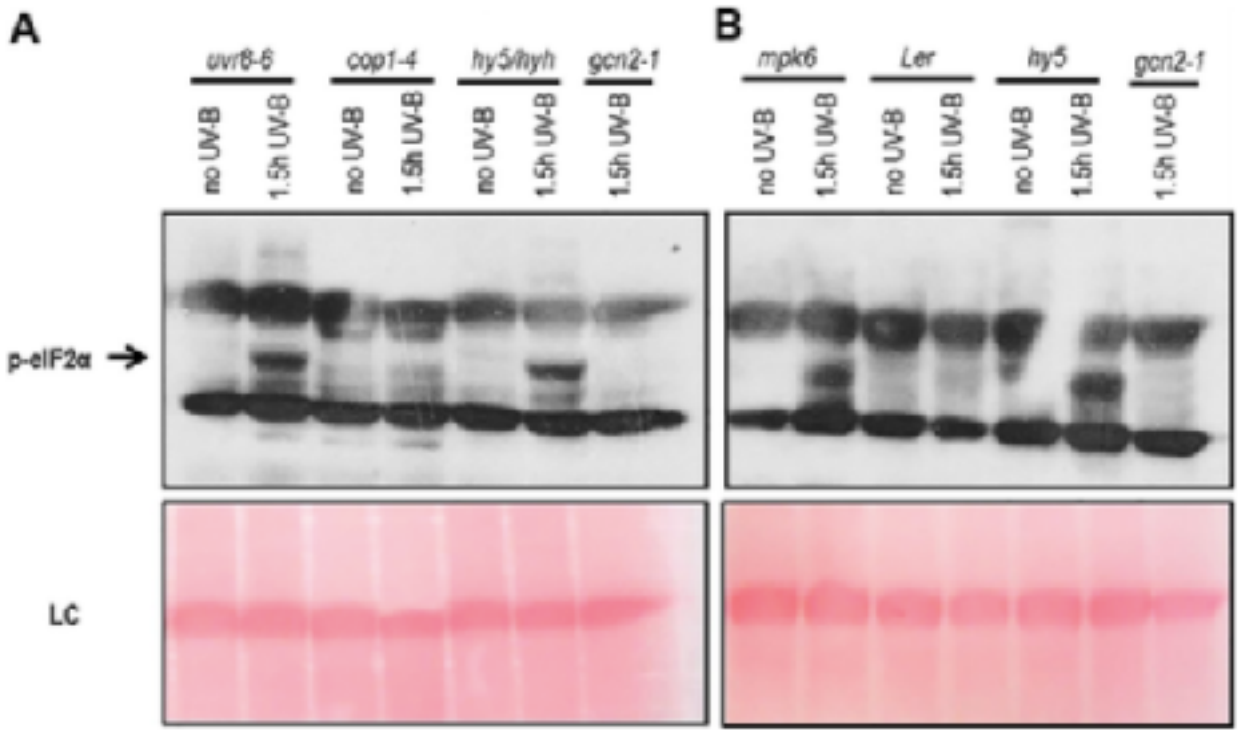

Figure 2. Western blots assaying activation of GCN2 via eIF2α phosphorylation in UV-B specific and stress signaling mutants. **(A)** Mutants of the UV-B photoreceptor specific signaling pathway and *gcn2-1* were harvested after 1.5 h of 6 µmol m^{-2} s^{-1} broad band UV-B treatment. **(B)** Wildtype and mutants of the stress and UV-B signaling pathway after1.5 h of broad UV-B with 10 µmol m^{-2} s-1. Equal amount of protein (20 µg) was loaded on 10% SDS-PAGEs. LC: Loading control.

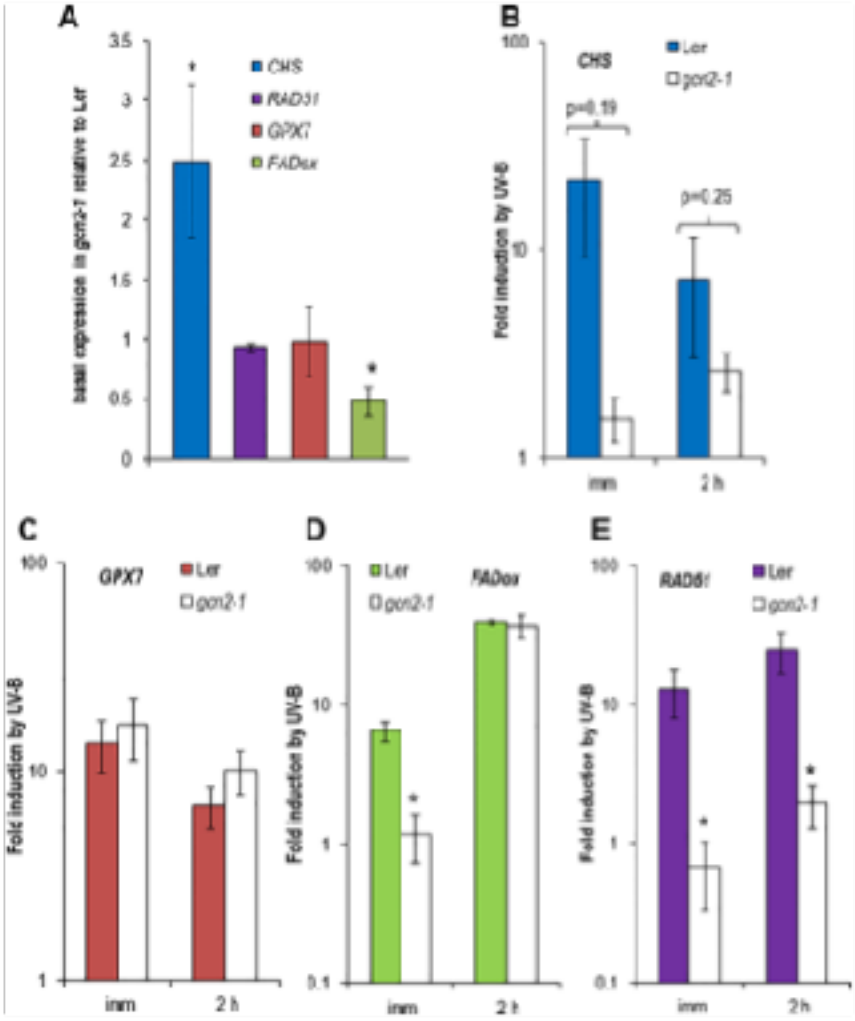

Figure 3. Expression of reporter genes dependent or independent of UVR8 and indicative for DNA damage in wildtype Ler and *gcn2-1*. In total, the expression of four different experiments (i.e. biological repeats) was quantified. Each cDNA was measured in triplicate. Data represent the mean and standard errors of the normalized expression to three reference genes. **(A)** Basal expression relative to the wildtype Ler using normalized data of three reference genes. (B - E) Fold induction by UV-B immediate and 2 h after a 1.5 h exposure to broad band UV-B of (B) the UVR8 dependent *CHS*, **(C)** the UVR8 dependent *GPX7*, **(D)** the UVR8 independent *FADox* and **(E)** the DNA damage induced *RAD51*. Stars indicated significant different expression to wildtype.

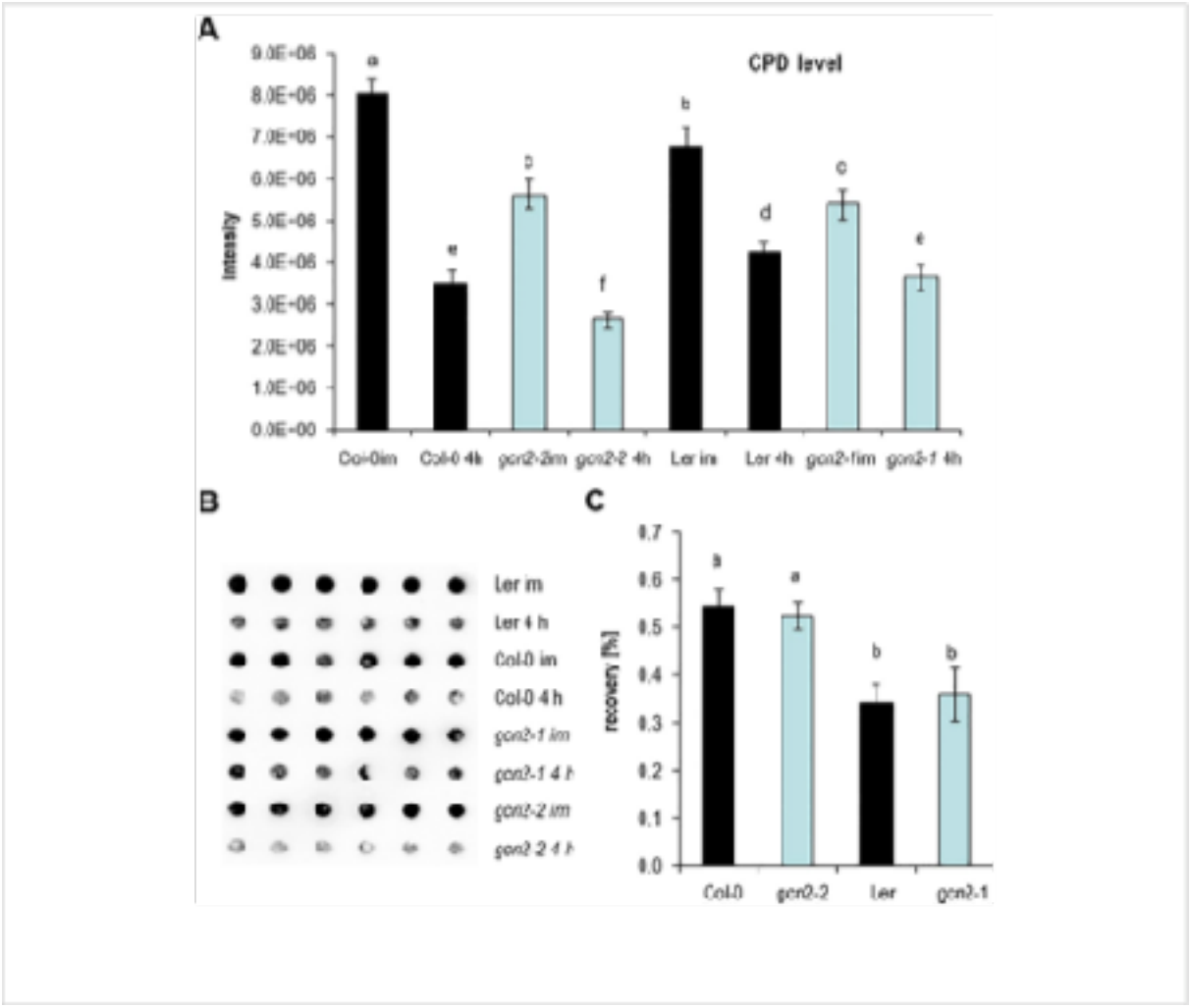

Figure 4. DNA damage and repair analysis. **(A)** Absolute CPD levels of plants harvested immediately after 1 h UV-B exposure and 4 h after UV-B shut down. **(B)** Representative image of a genomic DNA dot blot hybridized with anti-CPD antibodies. **(C)** Calculated recovery after UV-B exposure. Graphs represents means and ± SE of three to five experiments (six dots per experiment). Letters indicate the significant differences.

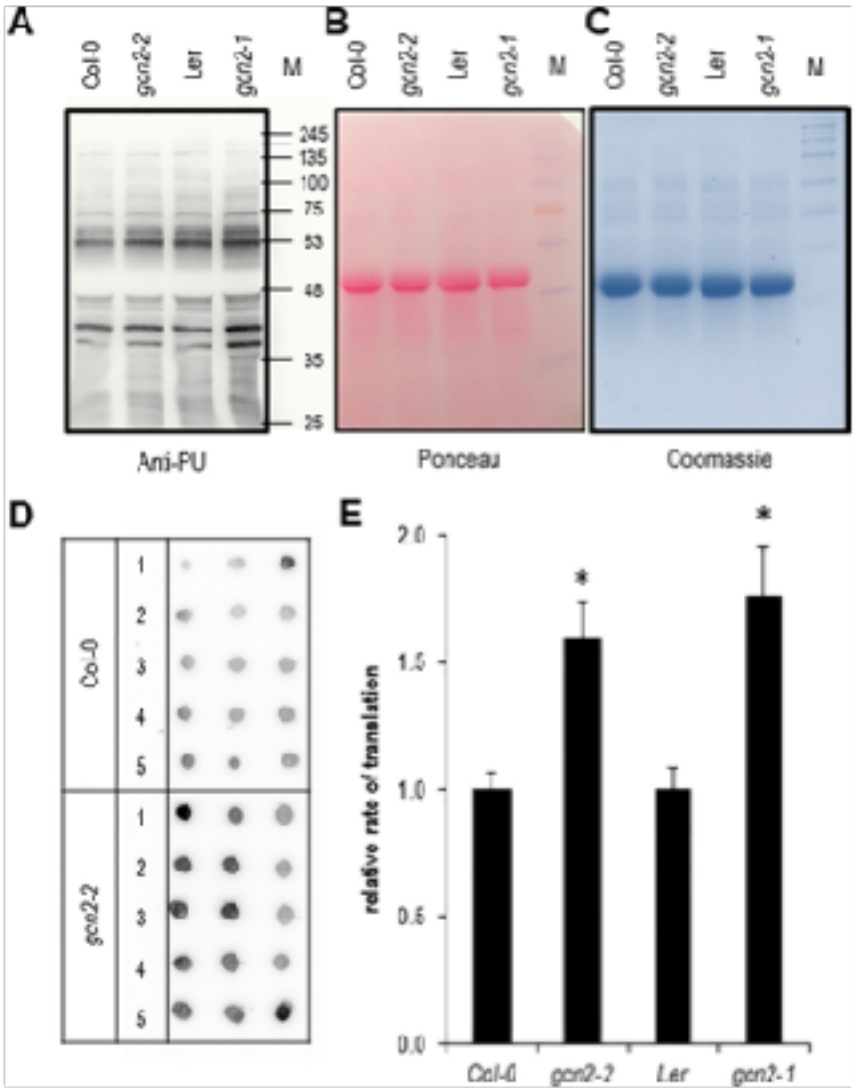

Figure 5. Quantification of the rate of protein biosynthesis using puromycin (PU) **(A)** Anti-PU Western blot with wildtype and *gcn2* mutants. To confirm equal loading of the 10% SDS-PAGE, the membrane was stained with **(B)** Ponceau S and **(C)** Coomassie. **(D)** Example of a dot blot analysis with total protein extracts of PU treated seedlings where PU incorporation was detected with anti-PU antibodies. **(E)** Quantification of the dot blot analyses**.** Bars represent means and SE of at least 15 dots per experiment. Data of Col-0 and *gcn2-2* are means of three independent experiments, data from Ler and *gcn2-1* from a single experiment. Stars indicate significant differences ($p<0.05$) between wildtype and mutants calculated with Student's t- test. M designates the protein size marker line.

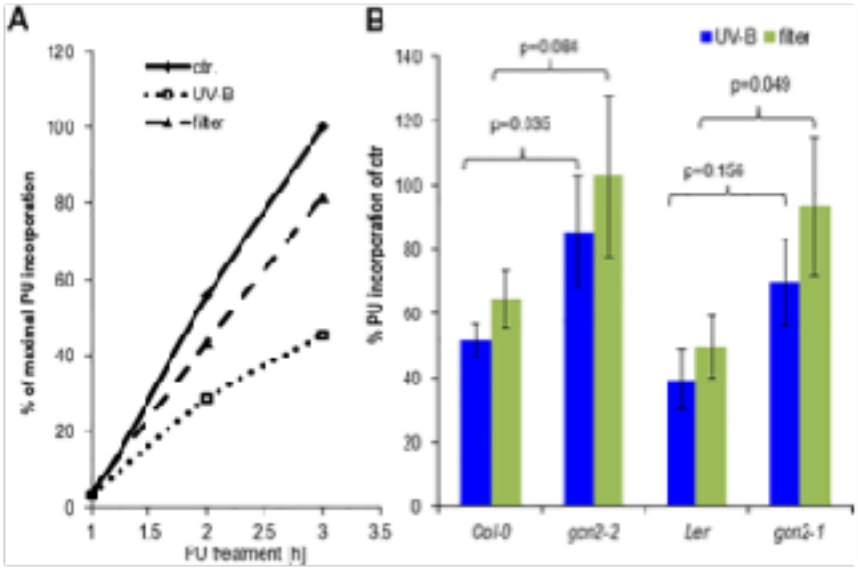

Figure 6. Effects of cellulose acetate filtered and unfiltered UV-B radiation on protein biosynthesis. **(A)** Time course of PU incorporation of detached rosette leaves. 100 mg/mL PU was added at the onset of a 1 h UV-B treatment. Control leaves were only exposed to white light. Leaves were harvested either immediately after UV-B shut down which correspond to 1 h, 2 h and 3 h after PU addition. (**B)** Comparison of PU incorporation of wildtype and gcn2 mutants upon UV-B exposure with filtered and unfiltered broad band lamps. In these experiments 100 µg/mL PU was added immediately after UV-B shut down and plant material harvested 1 h later. The graph represents the results of at least three independent Western blots. Error bars represent SE. P-values of student‘s t-test are indicated above the brackets.

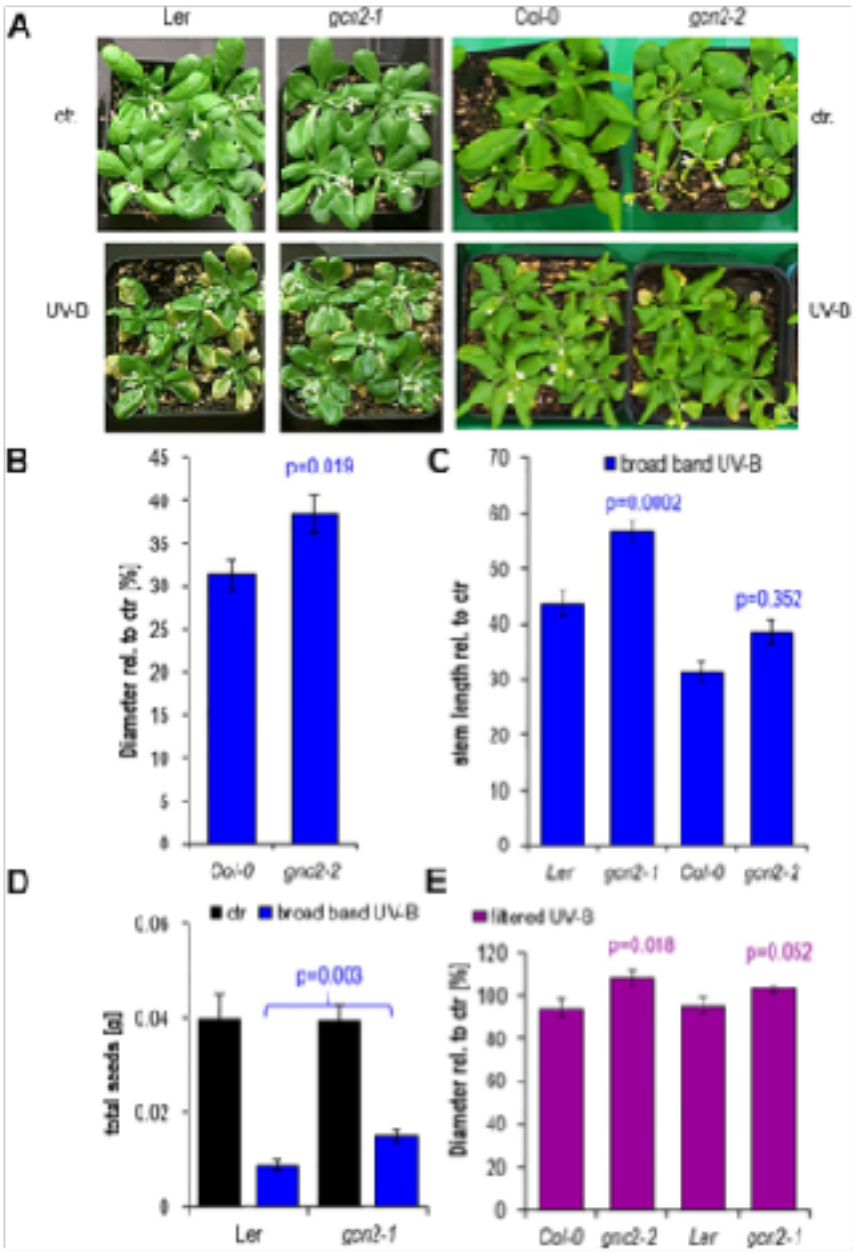

Figure 7. Phenotypes after 15 days of chronic UV-B exposure. **(A)** Rosettes of wildtype and *GCN2* mutants after a 1 h daily dose with 6 µmol m^{-2} s^{-1} UV-B broad band UV-B. **(B)** Quantification of rosette diameters of 18 to 21 plants. **(C)** Quantification of the stem lengths of 9 to 20 plants. **(D)** Quantification of the total seed weight of 9 to 19 plants. **(E)** Quantification of rosette diameter under chronic filtered broad band UV-B of 10 to 25 plants**.** Bars represent means and SE. Statistical differences between wildtype and mutants were calculated with Student's t- test and p-values are indicated above each bar. Numbers of plants are designated in the respective bars.

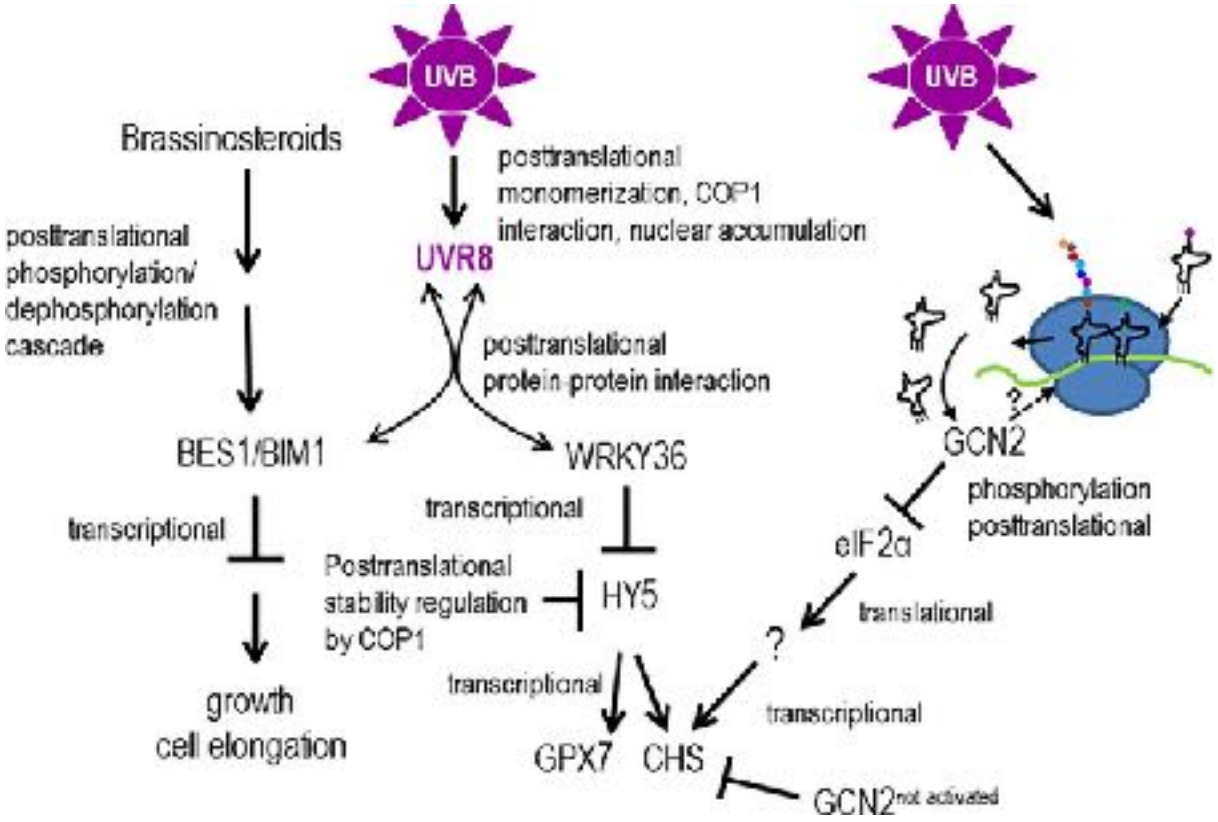

Figure 8. Models hypothesizing, how GCN2 might regulate UV-B responses. UV-B responses are mediated by transcriptional and (post)translational control involving the UV-B photoreceptor UVR8 or not. UV-B triggers UVR8 monomerization, interaction with COP1 and changes of the subcellular compartment. In the nucleus, UVR8 directly interacts with the downstream transcription factors BES1/BIM1 and WRKY36. Interaction with BES1/BIM1 and WRKY36 titrates these transcription factors away from promoters of genes involved in cell elongation and growth and allows HY5 to bind to its own and UV-B responsive promoters. Unbound COP1 destabilizes HY5. UV-B induces posttranslationally the activity of GCN2 which might be activated through structural changes of ribosomes by RNA-protein crosslinking, overaccumulation of uncharged tRNAs or direct ribosome binding. Activated GCN2 phosphorylates eIF2α and thereby changing the population of translated mRNAs. Not activated GCN2 might indirectly repress the transcription of energy demanding biosynthesis genes such as *CHS*.