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Identificacón genómica de genes traducidos ante situaciones de estrés en plantas

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Abstract

In response to amino acid starvation, general protein synthesis is repressed coincident with the increased translation of specific mRNAs such as those encoding the transcription activator GCN4 in yeast. Numerous genes involved in amino acid biosynthetic pathways controlled by the transcription factor GCN4 are activated for cell survival. This general amino acid control response (GAAC) is induced by the activation of the elF2α kinase, GCN2. Binding of GCN2 to GCN1 is required for stimulation of GCN2 kinase activity by uncharged tRNAs in starved yeast cells. Here, we shown there is a GCN1 homolog in *Arabidopsis thaliana.* Overexpression of the C-terminal segment of AtGCN1 in constitutively active GCN2 cells ($GCN2^c$) restored the defected cells growth characteristic of this strain, putatively by impairing association of yGCN1 to yGCN2. Overexpression of the same segment of AtGCN1 in wild-type cells under stresses shown no significant difference in phenotype in comparison to control cells, but the level of transcription of *GCN4* mRNA decreased. Furthermore, the YIH1/IMPACT like protein AT1G51730 found in *A. thaliana* also strongly lowered *GCN4* mRNA transcription. AT1G51730 contains the RWD domain, which binds to native yGCN1 and reduces yGCN2 function. These findings provide evidences of the evolutionary conservation of the translational control mechanism regulated by GCN2 in plants, as reported in other eukaryotes.

Resumen

En respuesta al ayuno de aminoácidos, la síntesis general de proteínas se reprime coincidiendo con el aumento de la traducción de mRNAs específicos, como los que codifican GCN4, un activador de la transcripción en levadura. Numerosos genes implicados en rutas biosintéticas de aminoácidos controlados por el factor de trasncription GCN4 se activan para la supervivencia celular. El control general de aminoácidos (GAAC) es inducido por la activación de la quinasa de elF2α, GCN2. La unión de GCN2 a GCN1 es necesaria para estimular la actividad quinasa GCN2 por tRNAs descargados en células de levadura en ayuno. Aquí, hemos demostrado que existe un homólogo de GCN1 en *Arabidopsis thaliana*. La sobreexpresión del fragmento C-terminal de AtGCN1 en células con GCN2 constitutivamente activa ($GCN2^c$) mejoró el crecimiento defectuoso de estas células, posiblemente debido a que afectaba a la asociación de yGCN1 a yGCN2. Mientras que la sobreexpresión del mismo fragmento de AtGCN1 en las células de tipo salvaje bajo estreses no muestró diferencia significativa en el fenotipo en comparación con las células de control, el nivel de transcripción de mRNA de GCN4 sí disminuyeron. Por otra parte, la proteina AT1G51730 hallada en *A. thaliana*, similar a YIH1/IMPACT, también redujo fuertemente la transcripción de mRNA de GCN4. AT1G51730 contiene el dominio RWD, que se une a yGCN1 nativa y reduce las funciones de yGCN2. Estos hallazgos proporcionan evidencias sobre la conservación evolutiva del mecanismo de control de la traducción regulado por GCN2 en las plantas, similar a otras eucariotas.

Palabras claves/Key words: GCN1, GCN2, AT1G51730, AT3G60300, *Arabidopsis, Saccharomyces*

For Tino and Lola;

For my parents.

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ABREVIATIONS

ATF4: Activating transcription factor 4
Bp: Base Pairs
BLAST: Basic Local Alignment Search Tool
C-terminal: Carboxy-terminal
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
dNTPs: Deoxyribonucleotide triphosphates
EDTA: Ethylenediaminetetraacetic acid
eIF2: eukaryotic initiation factor 2
eIF2α: alpha subunit of eukaryotic initiation factor 2
eIF2B: beta subunit of eukaryotic initiation factor 2
HA: Hemagglutinin
IMPACT: Imprinted gene with ancient domain
Kb: Kilo base
LB: Luria-Bertani culture medium for bacterial
Leu: Leucin
GAAC: General amino acid control
β-Gal: Beta galactosidase
GCN: General control non-derepressible
GDP: Guanosine diphosphate
GTP: Guanosine triphosphate
mRNA: Messenger RNA
N-terminal: Amino-terminal
OD: Optical density
ONPG: Ortho-Nitrophenyl- β -galactoside
PCR: Polymerase Chain Reaction
SD: Synthetic Dextrose culture medium for yeast
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SCD: Synthetic Complete Dextrose culture medium for yeast
SOB: Super Optimal Broth culture medium
S.O.C: Super Optimal broth with Catabolite Repression culture medium
TB: Tris-Borate buffer
TE: Tris-EDTA
tRNA: Transfer RNA
Ura: Uracil
WT: Wile-type
YIH1: Yeast impact homolog
YPD: Yeast Extract-Peptone-Dextrose culture medium

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1. 1 Eukaryotic Translational Control During Nutrient Depletion

All living organisms are sensitive to stimuli, both internal and external. Some of them may trigger cellular stress response to minimize changes in the environment or to repair possible cell damage, in order to re-establish the cell homeostasis. Different stressors and different intensities of stresses induce different cellular responses, such as nutrient deprivation, oxygen shock, temperature shock or DNA damage. A rapid respond to changes in the cellular environment is vital for cell survival. Most cellular processes are catalyzed by proteins, which perform a vast array of functions in the cell. Therefore, it is essential to effect rapid changes in protein levels in order to overcome any stress. Cellular concentrations of proteins can be regulated at many levels including genomic DNA transcription, alternative splicing, mRNA localization, mRNA translation, post-translational modification of proteins and proteins degradation (Spriggs *et al.,* 2010). Among them, the translation control of existing mRNAs through translation initiation factors is a particularly important mechanism to adjust cellular gene expression pattern. Compared to transcriptional regulation, translational control allows cell to effect changes in protein levels more rapidly under stress condition, which is essential for stress response and cell survival. Protein synthesis can be divided into three main steps including initiation, elongation, termination and ribosome recycling. Most regulation occurs at the translation initiation stage, the rate-limiting step, and thus it is highly regulated by several mechanisms including modifications of the initiation factors (Holcik M and Sonenberg N., 2005; Sonenberg and Hinnebusch., 2009).

A continuous supply of amino acid allows the normal function of the cell. Deprivation of even a single essential amino acid in eukaryotic cells can lead to alternation in gene expression profile and a general shutdown of protein synthesis. Many proteins are involved in the mRNA translation in eukaryotic cells and can be a target for regulation. Phosphorylation of the α subunit of eukaryotic initiation factor 2 (elF2α) is one of the key control modules which allows cell to shift down the general translation initiation rate for stress adaptation (Voorma, 1983). In fact, elF2 is the only initiation factor conserved in all eukaryotes and the phosphorylation of elF2α is one of the most commonly used mechanisms for inhibiting global translation. elF2 is a heterotrimeric protein composed of three subunits called α, β and γ (Proud, 2001). In the first step in translation initiation, elF2 in its GTP-bound form interacts with initiator methionyl-tRNA ($tRNA_i^{Met}$) and together form a ternary complex (elF2-GTP-$tRNA_i^{Met}$), which is subsequently loaded onto the 40S ribosomal subunit (Schmitt *et al.,* 2010). During a next step, the GTP bound to elF2 is hydrolyzed to GDP and elF2 delivers $tRNA_i^{Met}$ to the small ribosomal subunit and induce the protein synthesis. After initiation of translation, elF2 is released from the ribosome in its inactive form, elF2-GDP. To enable eIF2 to participate in further translation initiation and to reform the active ternary complex, it needs to be recycled to the GTP-bound form again by its guanine nucleotide exchange factor (GEF), elF2B (Kimball, 2002; Sattlegger and Hinnebusch, 2009).

When elF2α is phosphorylated, it converts from a substrate to a competitive inhibitor of elF2B and sequesters elF2B into an inactive complex. Since nearly all mRNA translation begins with $tRNA_i^{Met}$, phosphorylation of elF2α reduces the global protein synthesis and therefore lowers the overall utilization of amino acids (Castilho *et al.,* 2014). In addition, the synthesis of proteins required for responding stress is increased through selective recruitment of ribosomes to specific mRNAs.

Several elements in the 5' and 3' untranslated region (UTR) of mRNAs, such as upstream open reading frames (uORFs), may be involved in this recruitment (Sriggs *et al.,* 2010). These mRNAs code transcription factors that increase the expression of numerous genes essential for overcoming the shortage of amino acid. For example, the transcriptional factors GCN4 in yeast or ATF4 (Activating Transcription Factor 4) in mammal. *GCN4* mRNA contains four uORFs and induce synthesis of key enzymes in the amino acid biosynthesis pathway, as well as the amino acids transporters (Castilho *et al.,* 2014). When amino acids are abundant, those four uORFs of *GCN4* mRNA impede the flow of scanning ribosomes to the *GCN4* initiation codon. But when amino acids are scarce and elF2α is phosphorylated, ribosomes are able to bypass the most inhibitory uORFs and start translating *GCN4* mRNA (Marton *et al.,* 1993). The increased expression of GCN4 and its target amino acid biosynthetic genes occurring in response to amino acid starvation is known as the general amino acid control (GAAC) (Hinnebusch and Natarajan, 2002).

1.2 The elF2a Kinase: Gcn2

GCN2 (General control non-derepressible 2) is the sole elF2α kinase in *Saccharomyces cerevisiae,* required for cell growth during amino acid starvation. GCN2 is activated in amino acid starved cells and phosphorylates elF2α on its residue Ser^{51}. This phosphorylation reduces the general translation initiation by competitively inhibiting the guanine nucleotide exchange factor for elF2 (elF2B) and stimulates the translation of the transcriptional activator GCN4. In this way, GCN2 lowers the overall amino acid utilization and triggers the GAAC pathway to overcome the nutrient deprivation stress. GCN2 is activated through the binding of uncharged tRNAs, which is highly accumulated when cells undergo amino acid starvation (Dong *et al.,* 2000).

Unlike *S. cerevisiae,* who has only one elF2α kinase, there are four elF2α kinases in mammalian cells and each of them is activated by a different kind of stimulus: HRI (heme-regulated inhibitor kinase) by heme deprivation; PKR (dsRNA-dependent protein kinase) by double-stranded RNA produced during viral infection; PERK (endoplasmic reticulum transmembrane protein) or PEK (pancreatic elF2α kinase) by endoplasmic reticulum stress; and EIF2AKA, the mammalian homolog of the yeast GCN2, is also activated by amino acid deprivation (Wek *et al.,* 1989; de Haro *et al*., 1996; Berlanga *et al.,* 1998).

Yeast GCN2 is a 1659 amino acids long protein and it is composed of 5 different functional domains. The first one is the RWD domain (RING finger proteins,WD-repeat-containing proteins and yeast DEAD-like helicases) in the N-terminus, this domain is responsible for the interaction with its effector protein GCN1 (General control non-derepressible 1) *in vivo*. The association with GCN1 is important for sensing the amino acid starvation and leads to the stimulation of the kinase domain of GCN2. Second, a degenerated kinase domain with no enzymatic function (ΨPK). Nevertheless, this pseudokinase domain is still required for GCN2 kinase activity both *in vivo* and *in vitro*, suggesting that it may has a regulatory function through interaction with the functional kinase domain. Third, the catalytic domain of the elF2α kinase (PK) located in its middle portion. Upon kinase activation, the binding of substrate elF2α is allowed and it is phosphorylated by GCN2 on the amino acid Ser^{51}. Forth, another domain, whose sequence shares homology with the Histidyl-tRNA synthetase (HisRS). The

uncharged $tRNA^{deacyl}$ accumulated under amino acid starvation condition is transferred from the ribosome to this HisRS-like domain in GCN2, activating the kinase domain, leading to GCN2 auto-phosphorylation and phosphorylation of its substrate elF2α. Finally, a C-terminus region (RB/DD or CTD), necessary for the GCN2-ribosome interaction and GCN2 dimerization. It should be noted that CTD region may has a second $tRNA^{deacyl}$ binding site or at least it promotes the binding of $tRNA^{deacyl}$ to the HisRS-like domain (Nameki *et al*, 2004; Hinnebusch, 2005; Sattlegge *et al*, 2011；Castilho *et al.,* 2014).

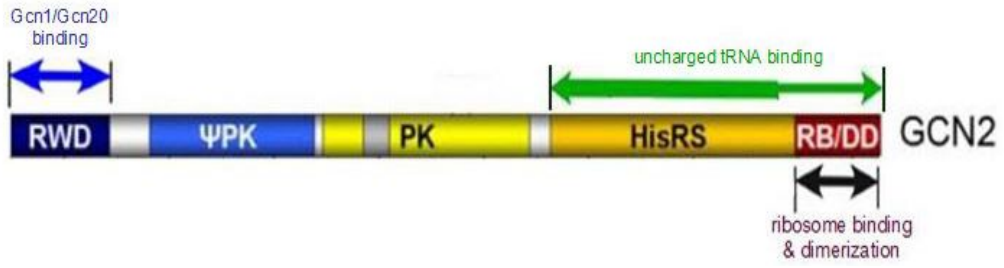

Figure 1.1. Schematic representation of yeast GCN2. GCN2 is composed of 5 functional domains, from the N-terminus to the C-terminus: RWD, psuedokinase (ΨPK), protein kinase(PK), HisRS-like (HisRS) and C-terminal(RB/DD) domains. Regions of interaction with GCN1/GCN20 complex, $tRNAs^{deacyl}$ and ribosome are indicated with double arrows. (Figure adapted from Padyana *et al.,* 2005)

Furthermore, three isoforms of *GCN2* RNA transcripts (α, β and γ) are identified in mouse with different expression profile in each tissues studies. Each of the these isoforms arises from a alternative promoters and has a unique translation initiation site but all finish translation at a common stop codon. The β *GCN2* mRNA is the most abundant one and is expressed in a variety of tissues. The N-terminus of the GCN2 β isoform contains the evolutionarily conserved RWD domain that interacts with GCN1 in yeast (Garcia-Barrio *et al.,* 2000; Kubota *et al.,* 2000). However, the α and γ isoforms are missing all or part of the GCN1 binding domain, suggesting that they may be activated by a mechanism independent of GCN1 (Sood R *et al.,* 2000; Zhang *et al.,* 2002).

1.3 GCN1

All data shows that *in vivo* GCN1 is absolutely essential for GCN2 to sense $tRNA^{deacyl}$ accumulation and therefore for its activation. More specifically, GCN1 binds to GCN2 through the RWD domain in GCN2 and promotes the transfer of $tRNA^{deacyl}$ from the ribosome to the HisRS-like domain in GCN2. GCN1 is not required for the GCN2 kinase activity in itself but is necessary for GCN2 activation in response to amino acid starvation (Marton *et al.,* 1993). In fact, studies shown that activation of the constitutively active GCN2 still requires binding to GCN1 (QIU *et al.,* 2002).

GCN1 is a high molecular weight protein with 2672 amino acids. The region comprises from amino acid 1330 to 1641 shares sequence similarity with the N-terminus of the fungal translation elongation factor 3 (eEF3), an ATPase that promotes the release of uncharged tRNAs from the E-site of the ribosome. The eEF3-like region constitutes a binding site for GCN20 (General control non-repressible 20), another effector of the GCN2 kinase (Vazquez de Aldana et al., 1995; Marton et

al., 1997)). Except of this central region, GCN1 lacks any significant homology with other proteins. GCN1 binds near the ribosomal A-site through the region comprised of amino acid 1-2052, the majority of the GCN1 protein, and the N-terminus is required for association with GCN2 (Kubota *et al*., 2000; Castilho *et al*, 2014).

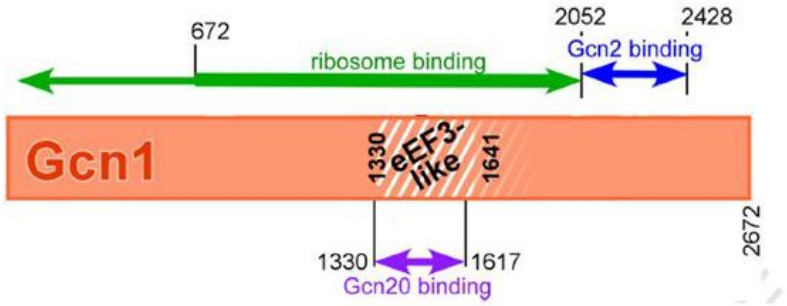

Figure 1.2. Schematic representation of yeast GCN1. Regions of interaction with ribosome, GCN20 and GCN2 are indicated with double arrows. The middle portion of GCN1 shows homology to the N-terminal HEAT repeat domain of eEF3. (Figure adapted from Castilho *et al*, 2014)

1.4 GCN2 Activation

GCN2 remains in its latent state under normal conditions and it is activated by $tRNA^{deacyl}$. Many stress conditions, including amino acid deprivation, lead to $tRNA^{deacyl}$ accumulation in cell, a cognate $tRNA^{deacyl}$ enters into the A-site of translating ribosomes, specially when the level of the cognate charged tRNA is very low. Then GCN1, GCN20 and ribosome form a trimeric complex that facilitate the transfer of $tRNA^{deacyl}$ from the A-site of the ribosome to the HisRS-like domain in GCN2. This interaction induces a conformational change in GCN2 that stimulates its catalytic domain, leading to its auto-phosphorylation. Activated GCN2 then phosphorylates the amino acid Ser^{51} in elF2α. On the one hand, phosphorylation of elF2α turns elF2α into a competitive inhibitor of elF2B and reduces the global protein synthesis and the overall utilization of amino acids. On the other hand, elF2α phosphorylation increases translation of specific mRNAs which encode transcription factors that control the translation of genes necessary for recovering from the shortage of amino acid. As a result, the GAAC control pathway is stimulated to regulate the cellular gene expression profile in response to stress (Fig. 1.3).

As mentioned before, GCN1 is crucial for GCN2 activation under amino acid starvation, since it is involved in the transmission of the starvation signal to GCN2. Studies show that overexpression of C-terminal GCN1 segment reduces elF2α phosphorylation and induce a dominant Gcn^- phenotype in amino acid starved cells. The truncated protein GCN1 binds to GCN2 through its C-terminus but it lacks the N-terminus for association with the translating ribosome and GCN20, therefore, the starvation signal can not be transmitted to GCN2. In other words, the truncated GCN1 competes with native GCN1 for GCN2 binding. GCN2 are sequestered, phosphorylation of elF2α decreases and a dominant negative effect is induced. Similarly, overexpressing the N-terminus of GCN2 produces the same effect. The truncated protein GCN2 interacts with GCN1 through the RWD domain and sequesters it. Truncated GCN2 competes with native GCN2 for GCN1 binding, the signal pathway is

interrupted and again, results in the dominant negative Gcn- phenotype. Nevertheless, this dominant-negative phenotype can be suppressed by overexpressing GCN2 or GCN1–GCN20 respectively, and the cell growth can be restored (Garcia-Barrio *et al.,*2000; Sattlegger and Hinnebusch, 2000).

Interestingly, the homologous N-terminal domain of Drosophila GCN2 also interacts with yeast GCN1-GCN20 and impairs cells growth under starvation conditions, suggesting that GCN1-GCN2 interaction is evolutionarily conserved (Garcia-Barrio *et al.,*2000).

1.5 YIH1/IMPACT

Two proteins have been described to be able to inhibit the GCN2 phosphorylation under amino acid starvation when they are overexpressed: BAA35139 or IMPACT (Imprinted gene with Ancient domain) in mouse (Pereira *et al*, 2005) and its homolog YCR059C or YIH1 (Yeast Impact Homolog) in *S.cerevisiae* (Sattlegger *et al*, 2004). The N-terminal domain of the yeast protein YIH1 and its mammalian homolog IMPACT share sequence homology with the GCN2 N-terminal RWD domain. In the C-terminus, the ancient domain is found throughout all kingdoms of life. Due to the similarity between the RWD domains, YIH1/IMPACT can bind to the C-terminus of GCN1, thereby prevent GCN1-GCN2 association and reduce GCN2 activation by competitive inhibition during amino acid starvation (Fig. 1.3).

Several studies show that, *in vivo,* overexpressed YIH1 suppresses elF2α phosphorylation and lowers the Gcn- phenotype in yeast cells, *in vitro,* it is demonstrated that purified C-terminus of GCN1 (amino acids 2052-2428) binds to purified YIH1. YIH1 interacts with GCN1 through its RWD domain and downregulates GCN2 kinase by preventing formation of the GCN1-GCN2 complex and therefore the elF2a phosphorylation by GCN2. Moreover, YIH1 overexpression can reverts the growth defect associated with constitutively active GCN2. Overexpressed YIH1 binds to GCN1, partially suppresses constitutively active GCN2 protein and impairs GAAC pathway. In addiction, overexpression of GCN2 from a high copy plasmid suppresses the Gcn- phenotype (Sattlegger *et al.,* 2004; Sattlegger *et al.,* 2011). All data prove that overexpressed YIH1 competes with GCN2 for the same binding site on GCN1, promotes the dissociation of GCN1-GCN2 complex, impairs GCN2 kinase activation and consequent inhibits the transcriptional control mechanism regulated by elF2α phosphorylation.

Similar results were obtained with IMPACT in mouse. IMPACT also regulates activation of the elF2α kinase GCN2 through its interaction with GCN1. In fact, IMPACT can inhibits both mouse and yeast GCN2. Furthermore, IMPACT depletion results in the activation of GCN2, increase of elF2α phosphorylation and inhibition of translation initiation. Also, overexpression of IMPACT inhibits GCN2 auto-phosphorylation and activation under stress conditions, GCN1-GCN2 interaction is disrupted by IMPACT, consequently leading to an impaired stress response (Pereira *et al.,* 2005; Roffé *et al*, 2013; Cambiaghi *et al*, 2014).

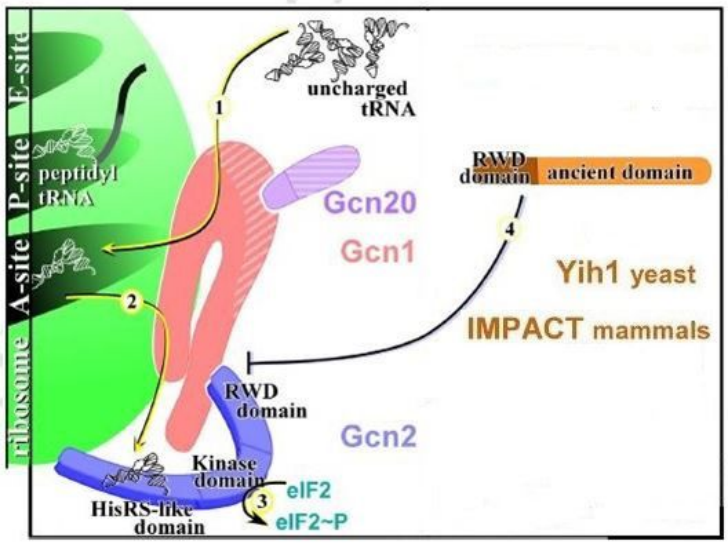

Figure 1.3. Working model for GCN2 activation by uncharged tRNAs and GCN2 inhibition by IMPACT and YIH1. Stress conditions including amino acid deprivation leads to accumulation of $tRNA^{deacyl}$ in cell, which constitutes a signal for GCN2 activation. 1) A cognate $tRNA^{deacyl}$ enters into the A-site of translating ribosomes. 2) GCN1/GCN20 complex transfers $tRNA^{deacyl}$ from the A-site of the ribosome to the HisRS-like domain in GCN2. 3) The catalytic domain in GCN2 phosphorylates elF2α and triggers responses for cell survival. 4) YIH1/IMPACT can downregulate GCN2 by competing with GCN2 for GCN1 binding through their RWD domains. (Figure adapted from Castilho *et al*, 2014)

1.6 GCN2 in *Arabidopsis thaliana*

Homologs of GCN2 have been identified in many organisms and they are implicated in diverse biological processes not directly related to the nutrient availability. All data suggests *Arabidopsis thaliana* only contain a sole GCN2-like eIF2α kinase. Plants respond to a large variety of biotic and abiotic stresses with GCN2, such as nutrient deprivation, herbicides, UV, cold shock or wounding, etc (Meurs *et al.,* 1990; Lageix *et al.,* 2008).

The AtGCN2 protein contain a eIF2α kinase and showed high sequence homology with the yeast GCN2 peptide sequence in the protein kinase domain. The protein kinase in AtGCN2 is structurally and functionally related to GCN2 and shares similar biochemical properties to its yeast and mammal homologs. Although compared to the yeast counterpart, AtGCN2 does not present a complete HistRS-like domain in it C-terminus, the binding of the $tRNAs^{deacyl}$ molecules to AtGCN2 was confirmed. $tRNAs^{deacyl}$ activates AtGCN2 leading to the phosphorylation of amino acid Ser^{51} in eIF2α, which is linked to a significant reduction in general protein synthesis. Moreover, the AtGCN2 kinase activity is conserved on both eIF2α homologs. AtGCN2 is shown to be activated in plants under amino acid deprivation conditions and it is able to restore yeast gcn2 mutant cells growth in the presence of an amino acids biosynthesis inhibitor. Overxpression of AtGCN2 in yeast yeast gcn2 mutants complements the mutation and reverts the impaired cell growth effect. (Zhang *et al.,* 2008; Li *et al.,* 2013; Zhang *et al.,* 2003).

2. Objectives

The main objective of this work is to elucidate some regulatory aspects about activation of GCN2 protein kinase in plants. In particular:

- ✓ the existence of a GCN1 homolog protein in *Arabidopsis thaliana* and if this protein can interacts with GCN2, as reported in other systems;

- ✓ the existence of regulatory proteins of GCN2 in plants similar to the YIH1 in *Saccharomyces cerevisiae* or IMPACT in *Mus musculus*:

For this purpose, a series of partial objectives were proposed:

1. Bioinformatic analysis to find out GCN1 homolog in *Arabidopsis* and proteins with RWD domain by which can interact with GCN2.

2. The design and synthesis of the C-terminal segment of *GCN1, AT1G51730* and *AT3G60300* for their appropriate insertion into the pENTR™/D-TOPO® plasmid and subsequently into the pAG425GAL-ccdB-HA plasmid by means of the Gateway® cloning system, as well as for their adequate expression in *E. coli* and the verification of the plasmid constructions integrity.

3. Transformation of two *S.cerevisiae* strains, wild-type and Gcn2^{c}, with the generated plasmid constructions: pAG425GAL-AtGCN1c-HA and pAG425GAL-A*T1G51730*-HA.

4. Immunoblotting assay to validate the correct expression of the proteins cloned in the destination plasmid.

5. Liquid growth assay to determine the response to amino acid-deprivation and acetic acid stress in the transformed *S.cerevisiae* strains.

6. ß-galactosidase enzyme assay to quantify the GCN4 expression in the transformed *S.cerevisiae* strains under amino acid- starvation and acetic acid stress conditions.

3.1 Strains and culture conditions

3.1.1 *Escherichia coli* (DH5α)

E. Coli DH5α strain (fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17) was used for the molecular cloning procedure of our genes of interest: C-terminus of *GCN1, AT3G560300* and *At1g51730.*

LB medium was applied for the the propagation and maintenance of *E.coli* cells. The medium was composed of tryptone 1% (m/v), NaCl 1% (m/v) and yeast extract 0.5% (m/v). The solid medium was prepared adding bacteriological agar 2% (m/v). For the growth and selection of transformed cells, the LB medium was supplemented with the corresponding antibiotic: ampicillin (50μg/ml), kanamycin (100μg/ml) or chloramphenicol (50μg/ml).

SOB medium was used for the obtaining of competent *E.coli* cells and it was composed of tryptone 2% (m/v), yeast extract 0.5% (m/v), NaCl 10mM, KCl 3mM, $MgCl_2$ 10mM and $MgSO_4$ 10mM. All *E. Coli* cells were grown at 37°C with agitation (180-230rpm).

3.1.2 *Saccharomyces cerevisiae* (W303)

The *S. cerevisiae* strains used in this work are listed in the following table. The genotype and origin as well as the wildtype strain and the mutations introduced in each strain are indicated in the Table 3.1:

Table 3.1. Characteristics of the *Saccharomyces cerevisiae* strains used in this work.

Strain	Genotype	Origin
W303-1A	MATa; ura3-1; his3-11,-15; leu2,3-112; trp1	EUROSCARF
W303::P180	W303.1A; p180::URA3	This laboratory, Aparicio-Sanchis,R
BY4741	MATa; his3Δ0; leu2Δ0; met15Δ0; ura3Δ0	EUROSCARF
GCN2^c	BY4741 GCN2^C-M719V-E1537G	Menacho-Marquez *et al.,* 2007

YPD medium was used for the competent *S. cerevisiae* cells preparation and contained yeast extract 1% (m/v), peptone 2% (m/v) and glucose 2% (m/v).

SD medium was used for the propagation and maintenance of *S. cerevisiae* and contained glucose 2% (m/v), Yeast nitrogen base 0,7% (m/v), succinic acid 50mM (pH=5,5 adjusted with Tris-Base).

SCD medium was prepared by adding a complete amino acids and nitrogenated base mixture which contained adenine 2,5mg/L, arginine 5mg/L; lysine 5mg/L, methionine 5mg/L, isoleucine 7.5mg/L, serine 7.5mg/L, threonine 7.5mg/L, tyrosine 7.5mg/L, valine 7.5mg/L, phenylalanin 12.5mg/L, inositol 21.25mg/L, p-aminobenzoic acid 22.5mg/L, tryptophan 25mg/L and histidine 25 mg/L. This SCD medium was used for propagation of the W303::P180 strain (SCD -Leu-Ura), while for *GCN2^c* the medium was also supplemented with 25mg/L of uracil (SCD -Leu). For SD and SCD medium, when required, glucose was substituted by raffinose 2% (m/v) or galactose 2% (m/v) as the alternative carbon source.

The solid medium was prepared adding bacteriological agar 2% (m/v). All *S. cerevisiae* cells were grown at 28°C with agitation (180-230rpm).

3.2 Plasmids

3.2.1 pENTR™/D-TOPO®

pENTR™/D-TOPO® is a 2580bp bacterial vector provided by Invitrogen™ (Fig.3.1) It is designed for a rapid and efficient directional TOPO® Cloning of blunt-end PCR products for entry into the Gateway®cloning system. The plasmid harbors a pUC high-copy replication origin for maintenance of the plasmid, *att*L1 and *att*L2 sites for site-specific recombination of the entry clone with a Gateway® destination vector, the directional TOPO® Cloning site, kanamycin resistance gene and M13 forward/reverse priming site for the transformed *E.coli* selection.

It is worth mentioning that in order to enable the directional cloning, the forward PCR primer must contain the sequence CACC at the 5' end of the primer. This 4 nucleotides sequence base pair with the overhang sequence, GTGG, in the pENTR™/D-TOPO® vetor. In this way, the directionality of the insert is ensured.

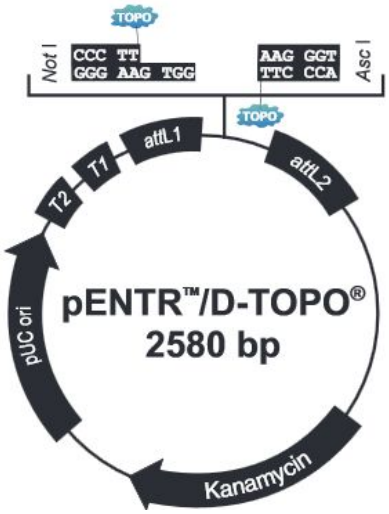

Figure 3.1. Graphic representation of the plasmid pENTR™/D-TOPO®. This plasmid was used as the entry vector in the Gateway®system. The location and the orientation of the kanamycin resistance gene and the pUC origin of replication are represented by arrows. The TOPO® directional cloning sites (att) are enlarged to show the exact location of the genes to be cloned.

3.2.2 pAG425GAL-ccdB-HA

pAG425GAL-ccdB-HA is a 9346 bp yeast expression vector provided by Invitrogen™ (Fig.3.2). It was used as the *Advanced Gateway Destination Vecto*r for a optimal expression of our genes of interest in *S. cerevisiae*. Some important features include: a galactose-inducible promoter (GAL1), three C-terminal hemagglutinin tags (HA), an auxotrophic markers (LEU), a ampicillin resistance gene, and a recombination site-flanked bacterial 'death' gene (ccdB), in this case, a chloramphenicol resistance gene. When the gene of interest is transferred from the entry vector to the destination vector, ccdB will be replace. All these markers facilitate the selection of colonies correctly transformed.

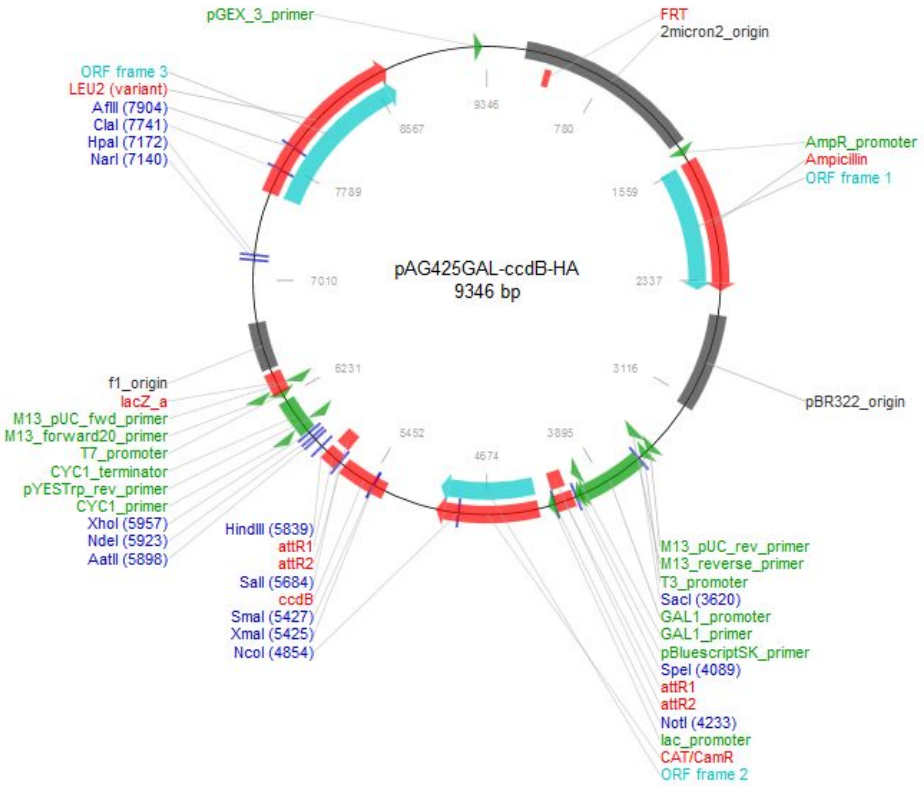

Figure 3.2. Graphic representation of the plasmid pAG425GAL-ccdB-HA. Destination vector in the Gateway® system. The GAL1 promoter is represented by green arrow. Additionally, the plasmid contains several selective markers used in this work, represented by red arrows: an ampicillin resistent genes, a chloramphenicol resistent gene and a leucine biosynthetic gene .

3.3 Primers

The primers used in this work are list in the following table. All of them have been designed with the primer design tool primer3.

Table 3.2. Oligonucleotide primers used in this work.

Primer	Sequence(5'→3')	Tm*(°C)	Amplification Target
F_GCN1overexp_n	CACCATGGCAGCTGTTCTTCCAC (23)	56.5	AtGCN1 (nt: 6334-8113)
R_GCN1NoSTOP	CCCACTTACATTATCATCATCGCTTTCA (28)	62.2	
F_AT1G51730	CACCATGACGGAGTATAAGCAG (22)	60.3	AT1G51730
R_AT1G51730	TGTCCTTGTTGGGGGAGC (18)	58.2	
F-AT3G60300	CACCATGACGGAGGAAGAGGTAGC (24)	66.1	AT3G60300
R_AT3G60300	TTTGTCTCCTTCTCTGGCTCTC (22)	60.3	
F_M13	GTAAAACGACGGCCAG (16)	51.0	pENTR™/D-TOPO®
R_CYC1_primer	GCGTGAATGTAAGCGTGAC (19)	56.7	pAG425GAL-ccdB-HA

*: Oligonucleotide melting temperature

3.4 DNA manipulation

3.4.1 Plasmid extraction

Plasmid DNA was extracted from 3mL of saturated culture of transformed *E.coli* cells in LB medium by means of the NucleoSpin®Plasmid/Plasmid (NoLid) (Macherey-Nagal) system. The procedure follows several principles: alkaline cell lysis and wash, DNA adsorption onto the NucleoSpin®Plasmid/Plasmid (NoLid) silica-gel membrane, removal of precipitated proteins, genomic DNA and others cells debris, and finally the purified plasmid DNA elusion. The DNA extracted was quantified with a NanoDrop® 1000 Spectrophotometer (Thermo Scientific) and the molecular weight was exterminated by a PCR amplification reaction and subsequently an agarose gel electrophoresis.

3.4.2 PCR amplification

Two different PCR reactions were performed in this work. For the selection of colonies successfully transformed with each plasmid constructions, the PCR amplification was carried out with Taq DNA Polymerases 1U/lµL (Biotools) in a final volume of 25µL. In contrast, for the extraction of the DNA fragment of interest, either from a previous PCR product or from the plant cDNA, TrueStart Hot Start *Taq* DNA Polymerase 5U/1µL (Thermo Scientific) was used. This DNA Polymerase present a proofreading mechanism thanks for its 3'-5' exonuclease activity. In this case, the PCR reactions were carried out for a final reaction volume of 50µL, having all reagents doubled (Table 3.3).

PCR reactions were carried out in a SureCycler 8800 Thermal Cycler (Agilent Technologies) . The program of each reaction was set according to the DNA polymerase, the primers and the length of the DNA fragment to be amplified (Table 3.4).

PCR products were analyzed by agarose gel electrophoresis. The length of the DNA amplified was confirmed using the molecular weight marker MassRuler™ DNA Ladder, Mix (Fermentas).

Table 3.3. PCR reagents and concentrations.

Reagents	Taq DNA Polymerases 1U/lµL	TrueStart Hot Start *Taq* DNA Polymerase 5U/1µL
DNA template (10ng)	1µL	1µL
DNA Pol. Buffer (10X)	2.5 µL	5µL
$MgCl_2$ (25mM)	-	5 µL
dNTPs mix (2mM)	0.5µL	5 µL
Forwar primer(10mM)	0.5µL	1 µL
Reverse primer (10mM)	0.5 µL	1 µL
DNA Polymerase	0.3µL	0.5µL
H_2O milli Q	to 25µL	to 50µL

Table 3.4. PCR program.

Step	Cycles	Taq DNA Pol.		TrueStart Hot Start *Taq* DNA Pol.	
		Temperature(°C)	Time (min)	Temperature(°C)	Time (min)
In. Denaturation	1	94	2	95	2
Denaturation	35	95	0.5	95	0.5
Annealing		60	0.5	60	0.5
Elongation		72	2	72	2
Final Elongation	1	72	5	72	5
Hold		4	-	4	-

3.4.3 PCR product purification

When required, PCR products were purified using GeneClean® *Turbo* kit (QBiogene, Inc). This system presents a membrane that optimizes the purification of DNA fragment with molecular weight between 0,1 and 300 kb. Since DNA binds to the membrane when the salt concentration is high and it is eluted when the salt concentration is low, the product can be eluted using water or low saline concentration buffer, therefore, the purified product is ready for further manipulation.

3.4.6 Directional TOPO® Cloning

The PCR products were cloned into the entry vector for the Gateway System with the pENTR™ Directional TOPO® Cloning Kits. The DNA ligation reaction reagents included: 0.5-4µL of fresh PCR product, 1µL of salt solution, 0.5µL of TOPO® entry vector and sterile water to complete the final volume of 5.5µL. The molar ratio of PCR product: TOPO® entry vector was 3:1 to maximize the cloning efficiency and to obtain the highest number of colonies. Once the reaction was prepared, it is gently mixed before incubation at 22°C for 20 minutes.

3.4.7 LR recombination reaction

The transfer of the insert from the entry vector pENTR™/D-TOPO® to the destination vector pAG425GAL-ccdB-HA was performed according to the Gateway® LR reaction protocol. 0,5µL-1µL of entry vector (150ng) was mixed with 0,5µL-1µL of destination vector (50ng). TE buffer (pH 8.0) was added to complete the volume of 4.5 µL. Then, to each sample, 1µL of LR Clonase™ II Enzyme was added to the reaction and it was mixed by vortex. The reaction were centrifuged briefly and they were was incubated overnight at 25°C. The next day, 1µL of Proteinase K solution was added to each sample to stop the reaction. After vortexing briefly, samples were incubated at 37°C during 10 minutes.

3.5 Competent cells preparation

3.5.1. Chemically competent *E.coli* cells preparation

Chemically competent *E.coli* cells preparation began by seeding an aliquot of glycerinated DH5α in LB solid culture medium. It is noteworthy that in order to ensure the absent of contamination, cells

were also seeded in LB solid medium supplemented with ampicillin (50µg/ml) and kanamycin(100µg/ml). After growing at 37°C during 24 hours, several *E.coli* colonies were incubated in 5mL of LB broth medium, negative control for LB contamination was incubated parallelly. Cells were grown at 37°C with agitation overnight.

Next day, 5mL of culture in stationary phase with OD_{600} between 1 and 3 were obtained. 400µL of this saturated culture were transferred to two flasks with 200mL of SOB medium respectively which were incubated at room temperature overnight until the OD_{600} was between 0.4 and 0.8. Both flasks were cooled on ice during 10 minutes and their content was transferred into 5 cold and sterile Sorvall centrifugation tubes. After centrifugation at 5000 rpm during 5 minutes at 4°C, the supernatant was removed and the pellet was resuspended in 10mL of cold TB (10mM Pipes, 55mM $MnCl_2$, 15mM $CaCl_2$, 250mM KCl.) and mixed by vortex. Then, all tubes were incubated on ice during 10 minutes and centrifuged again at 5000 rpm during 5 minutes at 4°C. Once eliminated the supernatant, the pellet was resuspended in 4mL of cold TB. Later the content of these 5 tubes were unified into a sole 15mL Falcon tube, cold TB was added to complete a final volume of 10mL. After that 700µL of DMSO was added slowly and the tube was shaked gently and continuously. All steps were carried on ice.

Eventually, cells were divided into 100µL aliquots in sterile and cold *eppendorf* tubes and were stored intermediate at 80°C.

3.5.2 Electrocompetent *E.coli* cells preparation

Electrocompetent *E.coli* cells preparation started by seeding an aliquot of glycerinated DH5α in a LB agar plate. After growing at 37°C during 24 hours, cells were incubated in a 50mL Falcon tube containing 20mL LB broth medium. Negative control for LB contamination was incubated parallelly. Cells were grown at 37°C overnight with agitation. In the following morning, 5mL of each saturated culture were transferred respectively into two 1L flasks with 250mL of LB broth medium. Incubation at 37°C on the shaker stop when the OD_{600} was between 0.4 and 0.6, which took approximately 2 hours and 30 minutes. Immediately flasks were put on ice for 15 minutes. After that, the content were transferred into two sterile 250mL centrifugation bottles, previously cooled. Centrifugation was carried out using the JLA rotor at 4°C and 6000 rpm for 10 minutes. The supernatant was removed and the pellet was resuspended with 250mL of cold H_2O in each bottle. Latter, another centrifugation at 4°C and 6000 rpm during10 minutes was carried out, the supernatant was eliminated and 50mL of cold glycerol 10% was added into each tube to resuspend the cells. After the third centrifugation at 4°C and 6000 rpm during 10 minutes and the supernatant removal, the pellet of each bottle was resuspended with 12.5mL of cold glycerol 10%. Then, the content of each bottle was transferred into two 50mL sterile and cooled Falcon tubes. Now, with the JA rotor, tubes were centrifugated at 4°C and 6000 rpm at 10 minutes. After eliminating the supernatant, pellets were resuspended with 1 mL cooled glycerol 10%.. Finally, cells were divided into 50µL aliquots in sterile and cold *eppendorf* tubes and were stored intermediate at 80°C.

3.5.3 Competent *S.cerevisiae* cells preparation

Electrocompetent *S.cerevisiae* cells preparation started by seeding an aliquot of each strain of glycerinated cells in YPD agar plates and incubating 28 ° C during 2 days (W303::P180) or 3 days (*GCN2*^{c}). Once cells were grown in the YPD agar plates, 3 or 4 colonies were picked up and

inoculated into YPD broth medium, 3mL for W303::p180 and 2mL for *Gcn2*^{c}. Cells were grown at 28°C with agitation overnight. The following steps were exactly the same for both yeast strains. Firstly, 100µL of saturated culture were transferred into a flask containing 100mL of broth YPD and then, cells were incubated at 28 ° C overnight on a shaker until the OD_{660} were between 0.6 and 1.0. Immediately, the content of each flask was divided into two 50mL sterile Falcon tubes and they were centrifugated at 2000 rpm during 5 minutes. After removing the supernatant, cells were resuspended in 5mL of LiTE 1x (0.1M Lithium acetate 1xTE, which contains 10mM Tris, pH 7.6, adjusted with HCL and 1mM EDTA). The second centrifugation was carried out under the same condition, 2000 rpm for 5 minutes. Latter, the pellets were resuspended with 1mL of LiTe, giving a final volume of approximately 2mL in each tube. The content of both tubes was mixed into only one 15mL falcon tube. After incubating at 37 ° C during 15 minutes, 213µL of ssDNA were added (0,49% of salmon sperm DNA, treated previously with sonification at 96°C for 10 minutes) and 400µL of glycerol 80%. Finally, cells were divided into 200µL aliquots in sterile and cold *eppendorf* tubes and were stored intermediate at 80°C.

3.6 Cell transformation

3.6.1 Chemically competent *E.coli* cells transformation

Once the C-terminus of *GCN1, AT3G60300* and *AT1G51730* are cloned into the entry vector pENTR™/D-TOPO®, chemically competent *E.coli* cells were transformed with theses plasmid constructions. 2µL of each Topo® Cloning reaction were mixed gently with 50µL chemically competent *E.coli* cells under sterile conditions. Plasmid pUC19 provided by the same commercial kit were used as transformation reaction control. Cells were incubated on ice for 30 minutes following by a 30 seconds heat-shock at 42°C without shaking. After that, tubes were transferred immediately to ice. 250µL of S.O.C. medium of room temperature were added and cells were incubated at 37 °C during 1 hour on a shaker. Then cells were spined down for 1 minute and resuspended in 150µL of LB medium. Finally, under sterile conditions, cells from each transformation were spread on a prewarmed selective plate and incubate overnight at 37°C. Cells successfully transformed with pUC19 were resistent to ampicillin, while those with pENTR™/D-TOPO® were resistent to kanamycin.

3.6.2 Electrocompetent *E.coli* cells transformation

One the LR recombination reaction was performed for the C-terminus of *GCN1, AT3G60300* and *AT1G51730,* electrocompetent *E.coli* cells were transformed. Under sterile conditions, 1 µl of each LR reaction was added into 50µl of electrocompetent *E.coli* cells. After mixing gently, cells were transferred into a electroporation cuvette, previously sterilized under UV light during 30 minutes. After incubated on ice for 10 minutes, electroporation were carried out (200 ohms, 25µF, 2.5kV and approximately 4 seconds). Immediately, 1mL of LB medium was added into the cuvette, cells were transferred into a sterile *eppendorf* tube and incubated at 37°C for 1 hour on a shaker. Cells then were spined down for 1 minute and resuspended in 100µL of SOB medium. Finally cells from each transformation were spread on a LB agar plate supplied with ampicillin and incubated overnight at 37°C.

3.6.3 Competent *S.cerevisiae* cells transformation

The procedure of yeast transformation was identical for both strains, both pAG425GAL-AtGCN1c-HA and pAG425GAL-*AT1G51730*-HA were transformed with the wild-type W303::P180 strain. On the other hand, only pAG425GAL-AtGCN1c-HA was introduced into the mutant strain Gcn2^{c}. Additionally, the pAG425GAL-ccdB-HA plasmid without any gene of interest was also introduced into both yeast strain. First of all, 100µL of competent cells were mixed with a volume of each plasmid construction corresponding to 600ng. Then, 600µL of PEG-LiTE 1x (8 volume of PEG 50%, 1 volume of LiAcTe 10x and 1 volume of sterile H_2O). Two incubation steps were carried out, the first one at 30°C during 30 minutes and the second one at 42°C during 20 minutes. After centrifugation for 1 minute at 11000 rpm, the supernatant was eliminated and the pellet was resuspended in 150 µL of H_2O. Eventually, each transformation were spread on a SCD selective plate with the appropriated selection maker. Cells were incubated at 28°C and the results were evaluated after 2 days for the wild-type strain and 4 days for the mutant strain *GCN2^{c}*. W303::P180 was grown in SCD -Leu -Ura medium, while *GCN2^{c}* was grown in SCD -Leu medium.

3. 7 Yeast protein extraction

Yeast total protein content was extracted by means of the Laemmli 2X loading buffer method. 3mL of saturated cells were centrifuged at 3000 rpm during 5 minutes, after the supernatant removal, the pellet was resuspended in 3mL of H_2O for washing. The pellet was washed again by centrifugation, cells were resuspended with 1mL of H_2O and then transferred into a 1.5mL *eppendorf* tube. Another centrifugation was carried out under the same condition, the pellets was resuspended now in 150µL Laemmli 2X (Laemmli 5X: 30% sucrose, 7.5% SDS, 0.01% bromophenol blue, 0.3 M Tris-HCl at pH 6.8 , 0.1M DTE and 10mM EDTA) at 95°C during 10 minutes for cell lysis. Finally, samples were centrifuged at 5000 rpm during 1 minute and were ready to used in the electrophoresis.

3.8 Electrophoresis and protein detection

3.8.1 PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a protein separation technique based on their differential rates of migration under the influence of an applied electrical field, which are inversely proportional to their molecular weight. The anionic detergent SDS can denature proteins in individual polypeptide chains and confer them negative charges. Thus, the electrophoretic mobility of proteins only depends on the molecular weight of the polypeptide chains. In this work, the Mini Protean 3 (BioRad) was applied. The gel matrix is composed of two parts: the *stacking* gel is cast over the top of the *resolving* gel. Proteins are concentrated in the stacking gel before entering the resolving portion of the gel, where the SDS-protein complexes separated according to their molecular weights. The composition of the *stacking* gel was 3% acrilamida:bisacrilamida 30:0.8, 0.1% SDS, 125mM Tris-HCl, 1% APDS, 0.1% TEMED and pH 6.8 and the composition of the *resolving* gel was 10% acrilamida:bisacrilamida 30:0.8, 0.1% SDS, 375mM Tris-HCl, 1% APDS, 0.1% TEMED and pH 8.8. First of all, 7mL of the *resolving* gel were loaded, followed by a layer of butanol to facilitate the

gel polimerization. One the *resolving* gel was polymerized, the butanol was eliminated and 3mL of the *stacking* gel were added. The gel was ready to used once the polymerization was finished.

Electrophoresis was carried out with SDS-PAGE Running Buffer 1X (SDS-PAGE 10X: glycin 1.92M, 1%SDS, pH8.8 adjusted with Tris), 20µL of sample were loaded into the gel together with the molecular weight marker PageRuler™ Prestained Protein Ladder (Fermentas). The gel was ran at 100V. Migration continued until the blue dye front was at the end of the glass plate.

3.8.2 Transferring to PVDF membrane

The transferring was performed with the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). First of all, a PVDF (Polyvinylidene fluoride) membrane was soaked in methanol for 30 seconds and then in TOWBIN transfer buffer (100ml SDS/PAGE 10x, 200ml methanol and 700 H_2O). The PVDF membrane, two fiber pads and four pre-cut Whatman papers were placed in a shallow tray filled with transfer buffer for a few minutes. Then the gel apparatus was disassembled and plates were pried apart. The stacking gel was cut off and soaked in the transfer buffer. The transfer apparatus was opened with the black panel lying flat on the bottom of the tray filled with transfer buffer and the transfer sandwich was prepared on it. First one fiber pad, then two Whatman papers, following by the SDS-PAGE gel, PVDF membrane, another two Whatman papers and finally one more fiber pad. Air bubbles were remove by rolling a glass tube on the membrane. One the transfer sandwich was prepared, the transfer apparatus was carefully closed and inserted into the electrode module, ensuring that the black panel facing the black cathode electrode panel. Bio-ice cooling unit was also inserted into the buffer chamber, which was filled with the buffer. Transfer was finish after 90min at 4°Cand 110V on a shaker.

3.8.3 Direct Blue 71 (DB71) staining

Direct Blue 71 (DB71) staining was carried out to visualize the proteins blotted onto the transfer membrane and to verify the transferring efficiency. This method is sensitive and fast (Hong *et al.,* 2000). For this purpose, DB71 stock dye solution (0.1% [w/v] DB71: 0.1g of Aldrich DB71 dye in 100mL of distilled water) was diluted. The membrane was stained with the diluted working dye solution (0.008%[w/v]DB71: 4mL of stock dye solution to 50mL of H_20) during 5 minutes on a shaker. After that, the membrane was washed with the washing solution (500mL of distilled water, 400mL of absolute ethanol and 100mL of glacial acetic acid) in order to eliminate the excess dye. Then the membrane was wrapped in plastic and scanned. Finally, it was destained with the destaining solution (350mL of distilled water, 500mL of absolute ethanol and 150mL of 1M sodium bicarbonate.)

3.8.4 Blocking and primary antibody hybridization

Membrane has been chosen for its ability to bind to both antibodies and the target proteins, that is why a blocking step is necessary to avoid non-specific interactions between the membrane and the antibody used for detection of the target protein. This was achieved by firstly incubating the membrane with the blocking solution (TBS1X-0.1% Tween 20 and 2% milk) on a shaker for 30 minutes at 37°C. Primary Anti-HA antibody was diluted with 10mL of blocking solution (dilution 1:5000) and the

membrane was incubated with the antibody on a shaker overnight at 4°C. The next day, the membrane was washed twice with the blocking solution and then once with TBS 1X on the shaker, 10 minutes each time.

3.8.5 Secondary antibody hybridization and Immunodetection

The membrane was incubated with a diluted secondary Anti-mouse antibody in 10mL of blocking solution (dilution 1:5000) on a shaker. After 1 hour, the membrane was washed three times with TBS 1X for 10 minutes each time, also on the shaker.

For the protein detection, ECL plus chemiluminescence detection system was applied. Reagents A and B were mixed in a 1:1 ratio (500µL of each solution). The solution was aliquoted onto the membrane and it was incubated during 5 minutes in darkness. After that, ECL solution was drained and the membrane was wrap in plastic and was exposed to photographic films also in darkness for the chemiluminescence signal visualization., during 1 minute, 2 minutes and 1 hour.

3.9 Liquid growth assay or Bioscreen assay

To perform the liquid growth assay, saturated yeast culture was diluted with the proper medium until the OD_{600} was 0.02. Samples were loaded into a 100-well microplates, with at least three replicates each sample, as well as the negative control of each medium. Then the yeast cells proliferation in broth growth medium was quantified by means of a Bioscreen C plate-reader (Thermo Labsystems). The OD was measured every 30 minutes during 72 hours, through a wide band filter (420-580 nm), which is used to measure turbidity because it is less sensitive to color changes. Cells were grown at 28°C and they were subjected to shaking for 30 seconds before each reading.

3.10 β-galactosidase assay

In order to determine the *GCN4* gene transcriptional activity, yeast cells transformed with the p180 plasmid were grown until the logarithm phase. Pellets of 6mL cells in mid-log phase (OD_{600}=0.6) were obtained. The OD measurement and volume used were recorded for the further calculation of the activity units. Cell pellets can be frozen at -70°C or used right away. 110µL of GTED (20% glycerol(v/v), 10mM Tris pH 7.6, 1mM EDTA pH8, 1mM DTT or DTE) were added to the pellets and resuspended throughly. 10µL of the mixture were used to to measure the OD_{600} 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 resuspended throughly by vortex for permeabilization of cell membrane. Then tubes were immediately put on ice. After that, in a 96 well plate, 5µL of cells were miexd with 95µL Z buffer (1M sodium phosphate, pH7, 10mM KCL, 1mM $MgSO_4$, 50mM β-mercaptoethanol(add fresh from concentrated stock)). The reaction started when 20µL of ONPG (4mg/ml o-nitrophenyl-beta-galactoside 13.3mM in 0.1 M sodium phosphate, pH7) were added into each tube (Time=0). The addition of the ONPG to each tube was stagger (i.e.every 10 seconds). Samples were incubated at 28°C until the yellow color developed (approximately 2 hours). Wells without cells were used as contamination control while wells without ONPG were reaction controls. The reaction were stoped with 50µL of Na_2CO_3 (1M) when the yellow color has developed (use the

same stagger as with ONPG). The time elapsed was recorded for the calculation of the activity units. Finally, the absorbance at 415nm was read in a Bio-Rad spectrophotometry.

Activity units were calculated according to the following equation:

(680 x A415)/(time x Vc x A660)= arbitrary units of β-galactosidase activity

Where: 680 is a correction factor specific for microtiter plate and β-galactosidase activity

Time is in minutes

Vc (volume of culture used) =equivalent volume in ml of culture used in each sample

Vc = 6mL total culture volume x (5μL culture each well/110μL GTED each well) = 0.272

4. 1 Bioinformatic analysis of protein structure

4.1.1 GCN1-GCN2 interacting domains

Previous studies demonstrated that GCN1 is required for GCN2 activation. More specifically, the C-terminal segment of GCN1, amino acids 2052-2458, binds to the RWD domain of GCN2 and promotes its function. Several researches carried out a experiment where different fragments of GCN1 were expressed and their GCN2 binding activity was tested in a protein interaction assay. The results they obtained indicated some C-terminal fragments containing amino acid 2052-2458 were able to bind to GCN2 in this assay. In addition, a large N-terminal fragment containing region A and part of region B also showed GCN2 binding activity, although it is relatively weak (Fig 4.1) (Sattlegger and Hinnebush, 2005).

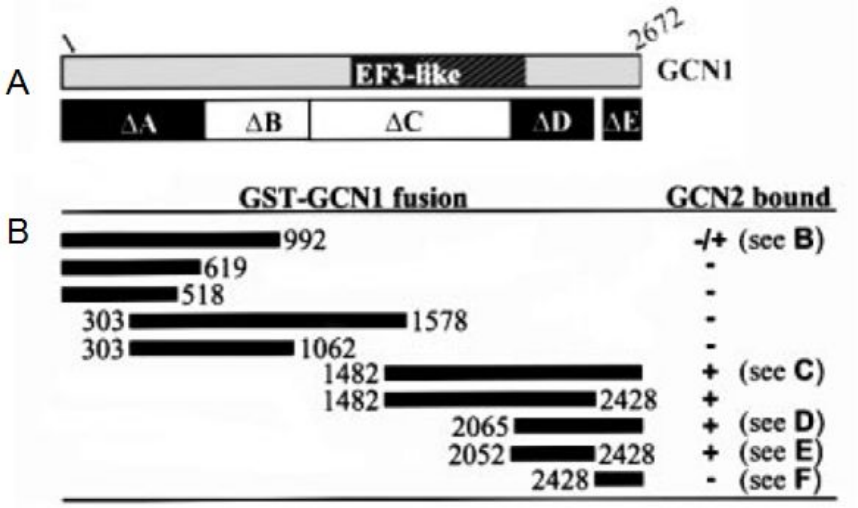

Figure 4.1. The C-terminus of GCN1 contains the critical GCN2-binding domain. A) Schematic representation of different domains of yeast GCN1. The regions highlighted in black show GCN2 binding activity. B) Result of protein interaction assays between GCN2 and different GCN1 fragments. Several C-terminal fragments containing region D strongly bind to GCN2, suggesting this region (residues 2052-2428) of GCN1 is sufficient for binding GCN2 in vitro (Figure from Sattlegger and Hinnebush, 2005).

As mentioned before, it was reported that the region D of GCN1 comprises the keys amino acids necessary for GCN1-GCN2 interaction in yeast. In this work, a bioinformatic analysis was carried out in order to find out the GCN1 homolog in *Arapbidopsis thaliana*. A BLAST search on the Arabidopsis genome shown that the only protein in *A.thaliana* that contains the conserved sequence was *AT1G64790* or *AtGCN1*, also named *ILITYHIA* (Fig 4.2). Studies in our laboratory out of the scope pf this TFG have shown that *Δgcn1* knock-out mutants plants are not able to phosphorylate elF2α, which contributed to demonstrate that AtGCN1 binds to AtGCN2 and promotes its activation (Faus *et al.,* unpublished).

A multiple sequence alignment of already characterized GCN2-interacting domains in GCN1 of different species was performed, including *Mus musculus*, *Homo sapiens*, *Saccharomyces cerevisiae, Neurospora crassa* and *Arabidopsis thaliana.* The multiple sequence alignment also shown that some

residues were particularly well conserved in all organisms analyzed, conforming a consensus sequence: ITGPLIR[bulky hydrophobic]$_2$ G[negatively charged] RF. In particular, the amino acid Arg2259 of yGCN1 is essential for high level GCN2 function *in vivo*. It is already known that R2259A single mutation in full-length GCN1 abolished the interaction between native GCN1 and GCN2 in yeast cells and destroyed GCN1 regulatory function (Sattlegger and Hinnebush, 2005).

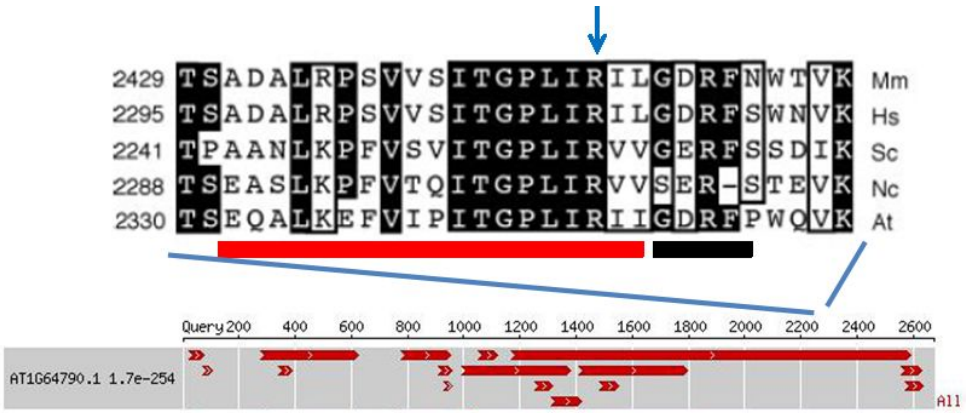

Figure 4.2. Multiple sequence alignment of GCN2-interacting domains in GCN1 of different species. Species studies included: *Mus musculus* (Mm), *Homo sapiens* (Hs), *Saccharomyces cerevisiae* (Sc), *Neurospora crassa* (Nc) and *Arabidopsis thaliana* (At). *AT1G64790* or *AtGCN1* is the only protein in *A.thaliana* with the keys amino acids for GCN2 binding. Consensus residues and sequences are in black, the highly conserved and critical residue Arg2259 (in yeast) is indicated with arrow.

Experiments carried out by other researchers demonstrated that in GCN2, the interacting domain with GCN1 is in its N-terminus, called the RWD domain. Protein interaction and co-immunoprecipitation assays demonstrated the region containing amino acid 1-272 in GCN2 comprises the minimal segment sufficient for association of GCN2-GCN1 (Kubota *et al.,* 2000; García-Barrio *et al.,* 2000). Moreover, structural studies of the RWD domain of the mouse GCN2 protein by heteronuclear NMR spectroscopy have revealed the characteristics of this novel protein-binning domain (Fig 4.3). RWD domain was named after three major RWD-containing proteins: RING finger proteins, WD-repeat-containing proteins and yeast DEAD-like helicases. The structure forms an α+β sandwich fold with an α-β-β-β-α-α topology and is characterized by a invariant YPXXXP motif forming a stable loop including a triple β-turn. A structural-based alignment shown RWD domain structure is well conserved among the GCN2 proteins in different species, suggesting the regulation of GCN2 through GCN1 is also evolutionary conserved (Nameki *et al.,* 2004).

Despite the apparent absence of sequence similarity, RWD domain shared partially structural homology with E2-ubiquitin-conjugated proteins (E2). Both RWD domain and E2 have the YPXXXP motif. However, substantial differences in structure of their C-terminus can be observed. Also, E2 is an enzyme while RWD domain mediates protein-protein interation (Tong *et al*. 1997; Nameki *et al.,* 2004).

Overexpression of the GCN2 RWD domain or region D in GCN1 impeded the GCN1-GCN2 association, impaired cell growth under amino acid starvation conditions and produced a dominant

Gcn- phenotype. All data prove GCN1-GCN2 interaction through these regions is required for GCN2 activation (Castilho *et al*, 2014).

Moreover, it was also reported what the RWD domain of *D. melanogaster* GCN2 can interact with *S. Cerevisiae* GCN1, giving evidence of the evolutionary conservation of this interaction (Garcia-Barrio *et al.,* 2000). In the same way, it was believed that *A.thaliana* GCN1 may also interact with *S. Cerevisiae* GCN2. In this project, the C-terminus of AtGCN1 that the truncated AtGCN1 protein can produce a dominant Gcn- phenotype due to its competition with native yGCN1 for yGCN2 biding.

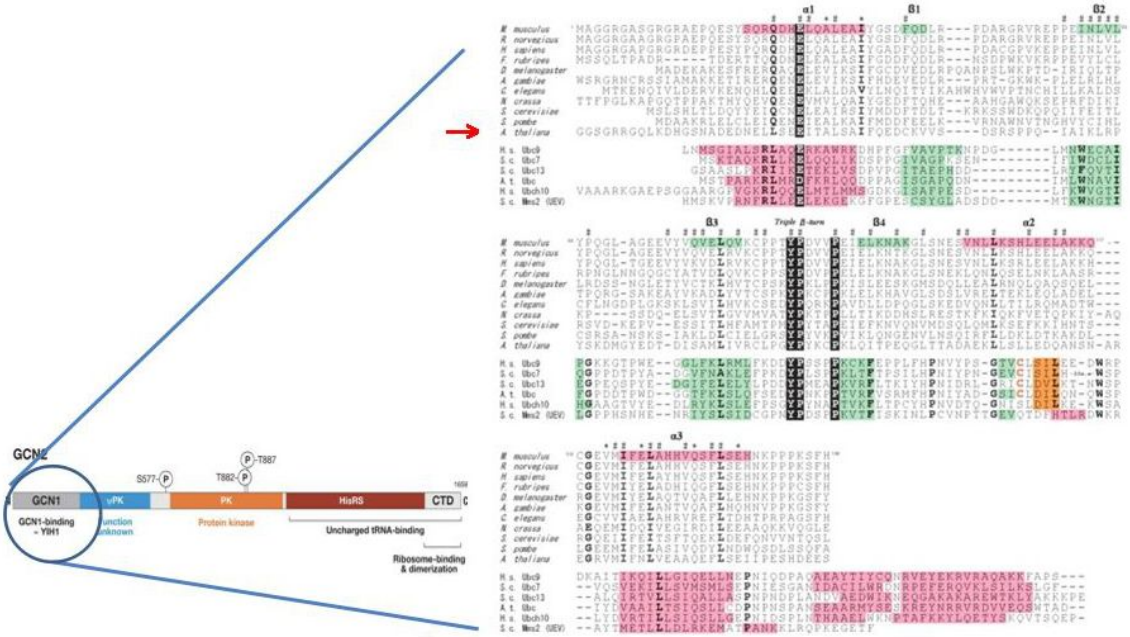

Figure 4.3. Structure-based sequence alignment of the RWD domain of GCN2 and the E2 family from various eukaryotes. α-helices, β strands and 3_{10}-helices are colored pink, green and orange respectively. The characteristic YPXXXP motif is indicated by a black background, *Arabidopsis* GCN2 is highlighted by a red arrow. (Figure adapted from Nameki *et al.,* 2004)

4.1.2 YIH1/IMPACT-like proteins in *Arabidopsis thaliana*

It was reported that there are several GCN1-binding proteins that shares the conserved RWD domain (and not belongs to the E2 family) with GCN2: the mouse imprinted gene IMPACT and its budding yeast homolog YIH1 in yeast. Indeed, the interaction region of GCN2 with GCN1 was named GI domain (GCN2 and-Impact) before discovering the RWD domain. Previous studies found out the conserved sequence among the homologs of GCN2, IMPACT and others, based on sequence alignment and further PSI-BLAST search (Kubota *et al.,* 2000).

It it shown that both IMPACT and YIH1 promote the dissolution of the GCN1-GCN2 complex. YIH1, when overexpressed, binds to GCN1 and suppresses elF2α phosphorylation by GCN2 producing the Gcn- phenotype (Sattlegger *et al.,* 2004). Similarly, overexpression of IMPACT in yeast cells inhibited cell growth under stress conditions in which GCN1 and GCN2 are required for survival.

Again, the fact that the mammalian IMPACT binds to the yeast GCN1 show the conservation of the GCN1-GCN2 interaction (Cambiaghi *et al.,* 2013).

What about *Arabidopsis thaliana*? In this work, a bioinformatic analysis was done to find out proteins with similar characteristics. The two YIH1/IMPACT-like proteins found in *Arabidopsis* are AT1G51730 or NP_175584 and AT3G60300 or NP_191589. By means of the structure-based alignment hh-pred (Söding, J et al, 2005), we demonstrate these proteins shared the RWD domain with homologs of GCN2 and IMPACT, especially secondary structures such as the three α-helices and the unique triple β-turn in the middle. For this reason, it was believed that yGCN1-yGCN2 interaction required for yGCN2 function may be disrupted by overexpression of AT3G60300 or AT1G51730 in yeast cells under stress conditions, AT3G60300 and AT1G51730 may compete with yGCN2 for yGCN1 binding through their RWD domains. We aimed to prove this hypothesis in this project.

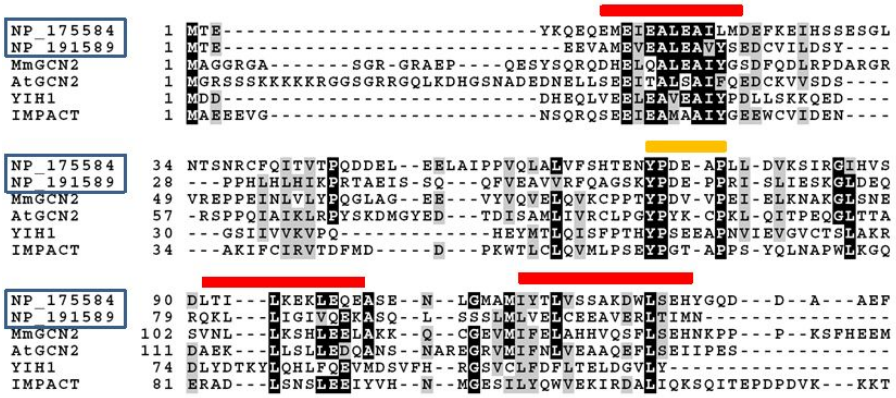

Figure 4.4 Sequence alignment of proteins containing RWD domain. α-helices are highlight by a pink line, while the triple β-turn formed by YPXXXP motif is indicated with the orange line. AT1G51730 (NP_175584) and AT3G60300 (NP_191589) shown sequence homology with homologs of GCN2 and IMPACT in yeast and mammalian.

4.2 AtGCN1c

4.2.1 Experimental design and plasmid construction

The following cloning strategy was designed in order to obtain yeast cells transformed with our plasmid constructions for further assays (Fig 4.5). The same cloning method was applied for all three genes.

First of all, the C-terminus of AtGCN1 (nucleotides 6334-811, hereafter AtGCN1c) was cloned by means of a PCR amplification reaction with the gene-specific primer (From now on this DNA fragment is named simply as AtGCN1). The forward primer contained the sequence, CACC, at its 5'end to enable the directional cloning. These 4 nucleotides base pair with the overhang sequence, GTGG, in the pENTR™/D-TOPO® entry vector. A cloned *AtGCN1* by a previous researcher in the laboratory was used as DNA template for our purpose,. An agarose gel electrophoresis was carried out in order to check the PCR product size (1780 bp) and then the PCR product was purified and the concentration was quantified (Fig 4.6.A).

Then At*GCN1c* was cloned into pENTR™/D-TOPO® by means of the Directional TOPO® Cloning Kits and the ligation product was transformed into *E. Coli* DH5α chemically competent cells. Transformed cells were seeded in LB agar plate supplemented with kanamycin. Since pENTR™/D-TOPO® contains a kanamycin resistance gene (Fig 3.1), only cells successful incorporated the plasmid construction could survive in the selective growth medium. The following day, colonies were picked up and were growth in LB broth medium supplemented with kanamycin untill saturation. Saturated culture was used to extract the plasmid applying the NucleoSpin®Plasmid/Plasmid (NoLid) (Macherey-Nagal) system. Then a PCR amplification with Taq polymerase and *AtGCN1* gene specific primers was carried out, following by an agarose electrophoresis to verified the insert size. One of the positive colonies with the highest DNA concentration was selected for further manipulation (Fig.1B). Additionally, another PCR amplification with M13 forward vector-based primer and *AtGCN1* specific reverse primer was performed to ensure the gene of interest *AtGCN1* was correctly cloned into the entry vector pENTR™/D-TOPO® .

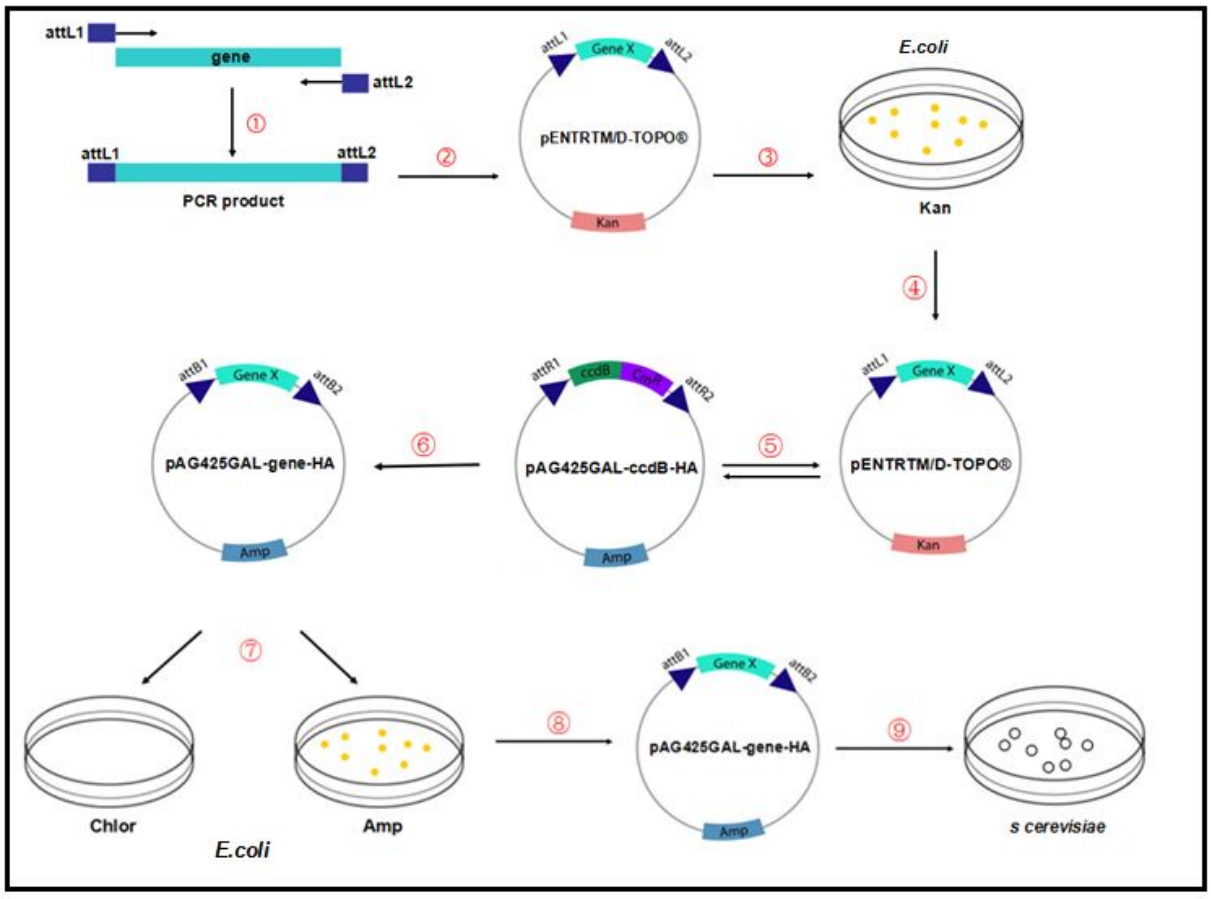

Figure 4.5 Overview of the workflow of the molecular cloning strategy. 1) Obtaining the gene of interest by PCR amplification. 2) Directional TOPO® Cloning of the gene into the entry vector. 3) Transformation of the chemically competent *E.coli* cells with the entry vector, positive clones were selected by kanamycin. 4) Plasmid extraction of positive colonies. 5) LR recombination to transfer the insert into the destination plasmid. 6) The destination plasmid was generated. 7) Transformation of the *E.coli* eletrocompetent cells with the destination vector, positive clones were resistent to ampicillin and sensitive to chloramphenicol. 8) Plasmid extraction of positive colonies. 9) Transformation of *S.cerevisiae* with the destination plasmid.

Once the construction integrity was verified, a LR recombination reaction was performed. *AtGCN1c* was transferred from the Gateway® entry clone pENTR™/D-TOPO® to the destination vector pAG425GAL-ccdB-HA. The LR reaction product was then transformed into *E. Coli* DH5α electrocompetent cells. Transformed cells were growth in LB agar plate supplemented with ampicillin. Since pAG425GAL-ccdB-HA contain a ampicillin resistance gene, only cells successfully incorporated the plasmid construction could survive in the selective growth medium. Colonies were picked up in the next day and they were grown in broth LB medium supplemented with ampicillin till saturation. Parallelly, colonies were also seeded in a LB agar plate supplemented with chloramphenicol, the bacterial 'death' gene (ccdB) in our destination plasmid is a chloramphenicol resistance gene. if the LR recombination reaction is successfully carried out, our gene of interest would substitute this gene and become sensitive to chloramphenicol. Plasmid was extracted from the saturated culture of a positive colony using the NucleoSpin®Plasmid/Plasmid (NoLid) (Macherey-Nagal) system. Next, a PCR amplification with *AtGCN1* gene specific primers was performed. The agarose electrophoresis shown that the *AtGCN1c* construct is cloned into the destination plasmid pAG425GAL-ccdB-HA and had the correct size (Fig.1C).

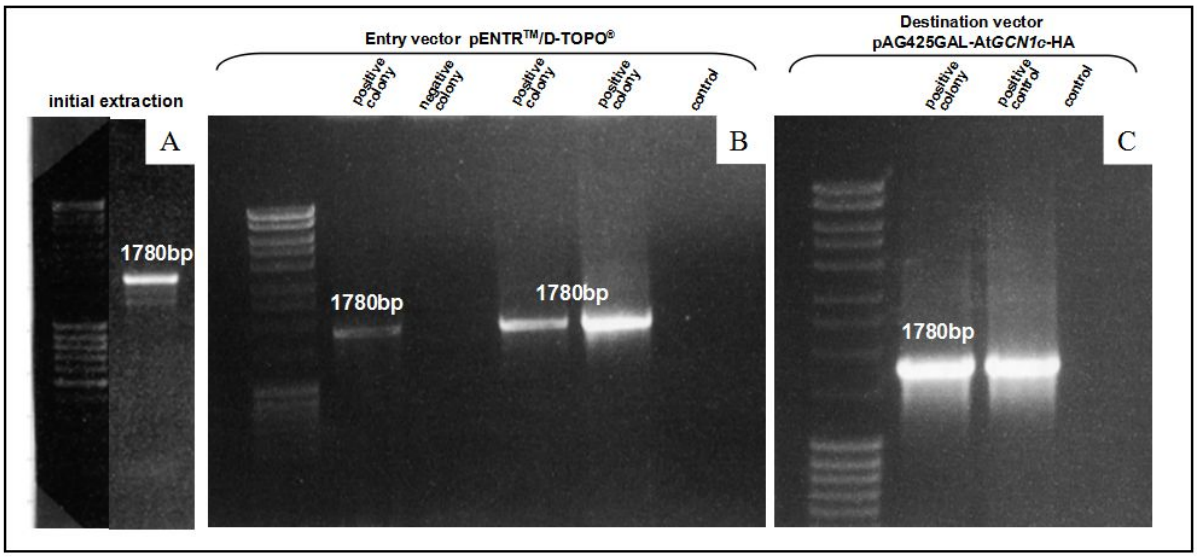

Figure 4.6. Gel electrophoresis of the PCR amplification of *AtGCN1c* with gene-specific primers. A) Extraction of *AtGCN1c* using as the DNA template a PCR product obtained by a previous researcher. B) Amplification of *AtGCN1c* inserted in pENTR™/D-TOPO® and screening for positive DH5α colonies. C) Amplification of *AtGCN1* inserted in pAG425GAL-AtGCN1c-HA, the positive control correspond to the pENTR™/D-TOPO® plasmid extraction before LR recombination.

Additionally, before transforming yeast cells, the inserted sequence of the new plasmid pAG425GAL-*AtGCN1c*-HA was verified through its DNA sequencing. Sanger sequencing was performed with the forward *AtGCN1* specific primer and the reverse vector-based primer CYC1. The result indicated that the inserted sequence correspond to our gene of interest *AtGCN1c* and had correctly incorporated three HA tags in frame in its C-terminus, which will be detected in the immunoblotting assay (Fig. 1).

pAG425GAL-AtGCN1c-HA

CAGGCTGCAGATTCTGGGGTACGTGAGGCCCATCCTATCTGCAATGAGAGGTGTAATAAAGCATGCTGGGAAGAGCATTGGGCT
GCTGTTAGAGTACGCATTTTTGATCTCCTAAAGGATCTAATGCACCATGAGGATGACCAAGTTCGAATATCTGCCACAAGCATGC
TGGGTGTTTTATCACAGTACTTGGAAGCTGCACAACTCAGTGTTCTGCTTCAAGAGGTCAATAATTTATCTGCTTCGCAAAATTG
GGGTGCTAGGCATGGCTCAGTTCTCTGCATTTCATCCTTGCTGAAACACAATCCTTCCACCATTATGACGTCATCGCTCTTTTCCT
CTATGTTAAATTCTCTTAAGAGCTCTTTGAAGGATGAGAAGTTTCCATTACGTGAAAGCTCGACAAAGGCCTTGGGAAGGCTTT
TGCTAAAACAACTTGCGACGGATCCTTCCAACACAAAAGTGGTTATCGATGTCCTTTCATCGATTGTCTCAGCTTTGCATGATGA
TTCGAGTGAGGTTCGAAGAAGAGCACTCTCTTCACTTAAAGCTTTTGCAAAAGATAATCCATCAGCAACCATGACAAATATAAG
TGTTATCGGACCTCCTCTAGCTGAATGTTTGAAGGATGGGAACACACCAGTGAGACTTGCTGCAGAAAGATGTGCTTTGCACGT
GTTCCAATTAACAAAAGGCGCAGAAAATGTTCAAGCAGCTCAAAAATACATTACAGGATTGGATGCCCGACGGTTATCCAAGTT
TCCTGAACAAAGCGATGACAGTGAAAGCGATGATGATAATGTAAGTGGGAAGGGTGGGCGCGCCGACCCAGCTTTCTTGTACA
AAGTGGTGATGGGCTGCAGGAATTCGATATCAAGCTTAGGTGGAATGTACCCATACGATGTTCCTGACTATGCGGGCTATCCCTA
TGACGTCCCGGACTATGCAGGATCCTATCCATATGACGTTCCAGATTACGCTGCTCAGTGCTAACTCAGAC

Figure 4.7. Sanger sequencing reads for pAG425GAL-AtGCN1c-HA.
Sequence underlined: Blue = Insert *GCN1* (uncompleted); Orange = three HA tags; Red = Stop codon.

4.2.2 AtGCN1 expression assay

The plasmid construction pAG425GAL-AtGCN1c-HA was introduced into two different yeast strains W303::P180 and *GCN2*^{c}. The first strain already contains a plasmid with a GCN4-LacZ reporter, which was used in the β-galactosidase assay, although it can be considered as a wild-type strain as well (from now on it named as WT strain). The second strain, *GCN2*^{c}, has two mutations in its GCN2 kinase protein: M719V in the kinase domain and E1537G in the ribosome association domain (Menacho-Marquez *et al.,* 2007). Yeast cells were also transformed with the empty plasmid pAG425GAL-ccdB-HA and they were used as negative control in each assay.

A western blot was carried out in order to check the expression of AtGCN1c in both strains, which was induced by galactose. All samples were grown firstly in presence of 2% raffinose till saturation and then in presence of 2% galactose during 4 hours. After that, the total protein content was extracted by means of the Laemmli method before performing the SDS-PAGE electrophoresis, 20 µl of each sample were used. AtGCN1c construct contains in its C-terminus 3 hemagglutinin (HA) proteins and they were detected by the anti-HA antibody in the immunoblotting. The detection of the HA tag protein provides information about the overexpression of AtGCN1 in both strains.

The results shown both WT and *GCN2*^{c} strains welly expressed AtGCN1c. The 72kDa bands in both strains corresponded to the molecular weight of the AtGCN1c protein plus 3 HA tags, while this band was not observed in the middle line, the control strain transformed with the empty plasmid. Some bands with lower molecular weights were also presence in WT and *GCN2*^{c} but not in the control strain, suggesting that they could be some degradation products of AtGCN1c(Fig.1).

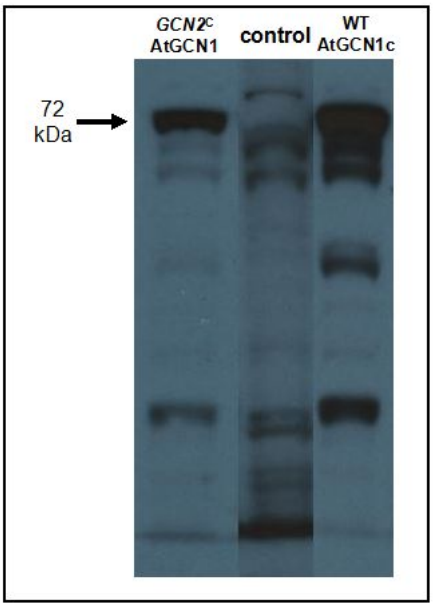

Figure 4.8. Immunodetection with Anti-HA antibody for overexpression of AtGCN1c cloned in WT and *GCN2^c^* yeast strain. Sample were treated with 2% galactose during 4 hours for induction. The WT and GCN2^c strains transformed with pAG425GAL-AtGCN1c-HA shown a 72 kDa band corresponded to the overexpressed protein, while the control strain was transformed with pAG425GAL-*ccdB*-HA, without insert, lacked this band characteristics fo AtGCN1. Some bands with lower molecular weight were also observed, they are putatively degradation products of AtGCN1.

4.2.3 *GCN2^c^*-AtGCN1 liquid medium assay

As mentioned before, it was reported by other researchers that the overexpression of the D region of yGCN1 or the RWD domain of yGCN2 impaired yeast cell growth and produced a dominant Gcn$^-$ phenotype. In this project, in order to find out if *A.thaliana* GCN1 can interact with *S. Cerevisiae* GCN2, the C-terminus of AtGCN1 was inserted into a proper expression vector, and the plasmid generated pAG425GAL-AtGCN1c-HA was introduced into a *GCN2^c^* yeast strain. The *GCN2^c^* strain contains two mutation in its GCN2 protein, M719V in the kinase domain and E1537G in the ribosome association domain. The GCN2 kinase is always activated in this strain and elF2α is constitutively phosphorylated in the absence of any stimuli such as amino acid deprivation (Menacho-Marquez *et al.,* 2007).

However, in this work we assumed that if the truncated protein AtGCN1c is expressed in this yeast strain and successfully interacts to yGCN2, AtGCN1c would compete with native yGCN1 for yGCN2 binding and the constituently active yGCN2 would be inhibited. Therefore, elF2α phosphorylation would decrease and the global protein synthesis would be restored. *GCN2^c^* cells overexpressing AtGCN1c would grow better than the control strain, *GCN2^c^* with the empty plasmid, pAG425GALccdB-HA.

To verify this hypothesis, a liquid medium assay or Bioscreen assay was performed by treating the transformed *GCN2^c^* with 2% galactose during 4 hours, for induction of the GAL promoter directing the expression of AtGCN1c. The results were the following: *GCN2^c^* overexpressing truncated AtGCN1

grown better than the control *GCN2*^{c} strain in galactose. In glucose medium, without the GAL promoter induced, the proteins was not expressed, and the difference between *GCN2*^{c} with pAG425GAL-AtGCN1c-HA and *GCN2*^{c} with pAG425GAL-ccdB-HA is minimal.

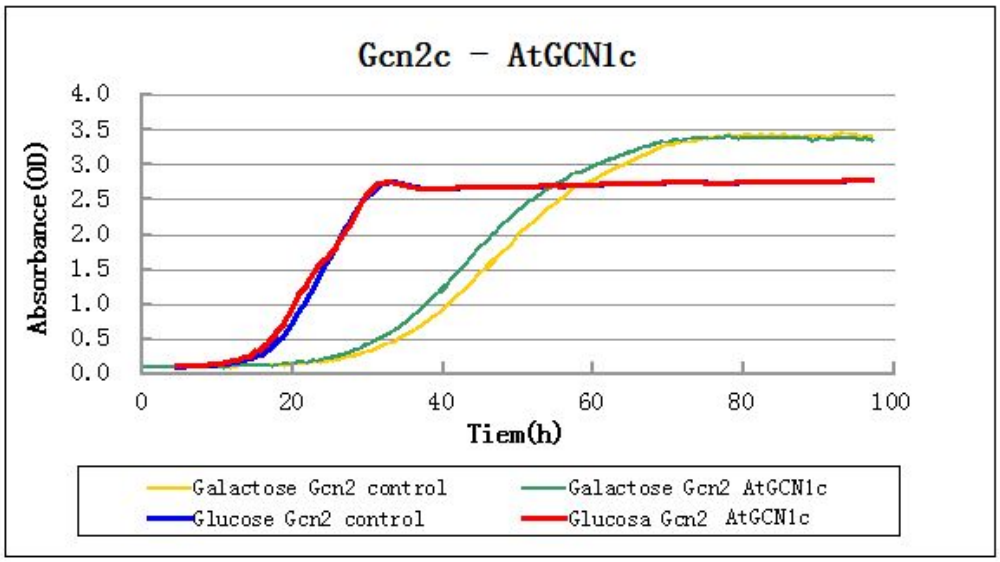

Figure 4.9. Growth curves of the control *GCN2*^{c} strain and *GCN2*^{c} overexpressing AtGCN1c. Both strains were grown in Galactose SCD-Leu medium and Glucose SCD-Leu medium. All samples were treated with 2% galactose before being analyzed in the Bioscreen reader.

4.2.4 WT-GCN1 liquid medium assay

The same experiment was carried out with the WT strain. In this case, since GCN2 is not constitutively activated, two different stresses were applied in order to activate the regulation mechanism governed by elF2α: amino acid starvation (SD medium comparing with SCD) and acetic acid stress (75mM and 100mM comparing with 0mM). As already discussed before, only the C-terminus of AtGCN1 is overexpressed, it would bind to native yGCN2 and reduce its activation under stress condition. In other words, overexpressed truncated AtGCN1 would sequester native yGCN2 and impede its function, therefore, the global protein synthesis would not decrease in the same way. Consequently, the WT yeast containing pAG425GAL-At*GCN1c*-HA should grow better than those with the empty plasmid pAG425GAL-ccdB-HA.

However, in both amino acid deprivation (Fig 4.11) and acetic acid stress (Fig 4.12) assay, there was no significant difference in growth between the control WT strain and WT strain overexpressing AtGCN1c, eventhough there are evidence from the previous western blot assay that the plasmid construction was correctly generated and the truncated AtGCN1 was welly overexpressed. The possible explanation would be the following: Although the truncated AtGCN1 was successfully expressed in yeast, and putatively bound to yGCN2 due to the evolutionary conservation of this mode of association, this interaction between AtGCN1 and yGCN2 is not strong enough to restore the defected cell growth caused by stresses applied. Some yGCN2 may be sequestered by AtGCN1, but the ones remain activated were sufficient to phosphorylate elF2α and trigger the downstream regulation pathway. That could be an explanation why the cell growth was not improved despite of the overexpression of the truncated AtGCN1.

A B

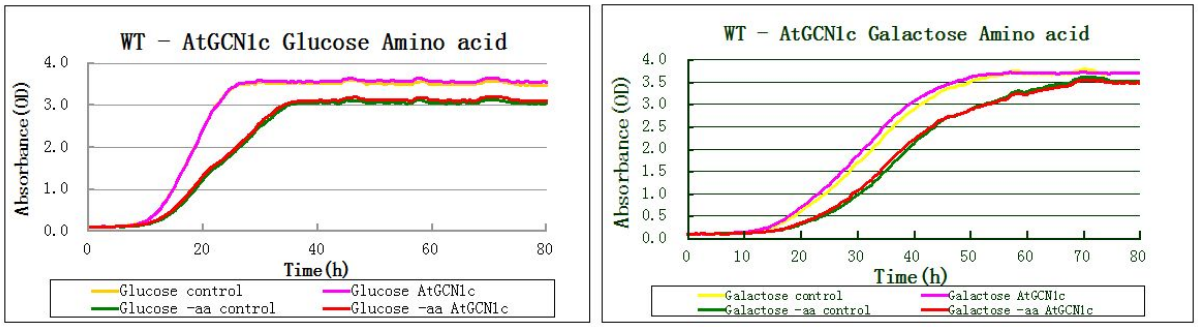

Figure 4.10. Growth curves of the control WT strain and WT overexpressing AtGCN1c under amino acid starvation. Both strains were grown in Glucose SCD-Leu-Ura medium, Glucose-SD-Leu-Ura medium (A) and Galactose SCD-Leu-Ura medium, Galactose SD-Leu-Ura medium(B). All samples were treated with 2% galactose before being analyzed in the Bioscreen reader.

A B

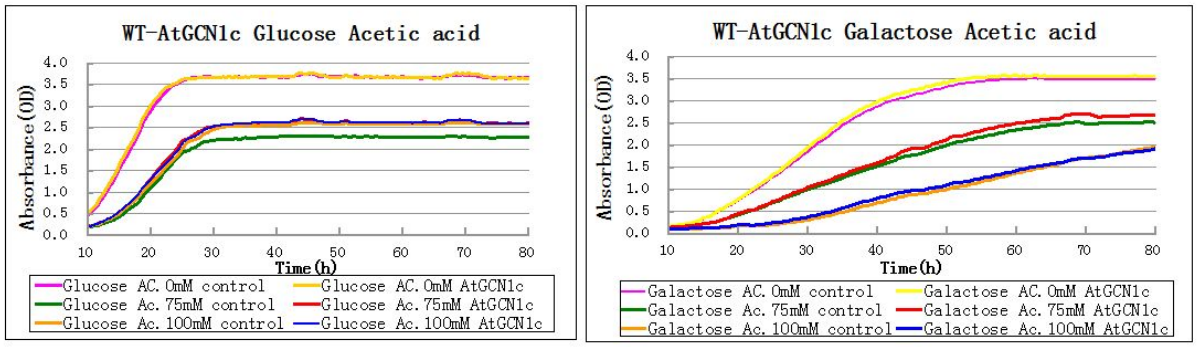

Figure 4.11. Growth curves of the control WT strain and WT overexpressing AtGCN1c under acetic acid stress (0mM, 75mM and 100mM). Both strains were grown in Glucose SCD-Leu-Ura medium(A) and Galactose SCD-Leu-Ura medium(B). All samples were treated with 2% galactose before being analyzed in the Bioscreen reader..

4.2.5 GCN4 expression assays

As seen above, no phenotypic differences were observed between the control WT strain and WT strain overexpressing AtGCN1c. To characterize this system more in detail, a β-galactosidase assay was performed to find out if overexpression of AtGCN1c had any effect on the translation of *GCN4* mRNA.

The WT strain (W303::p180) used in this work contains a GCN4-LacZ reporter which would provide evidence about the molecular difference between the control WT strain and WT strain overexpressing AtGCN1c. The overexpression of AtGCN1c should inhibit the elF2α kinase GCN2 and this will affect the translation of *GCN4* mRNA. Since the LacZ gene is downstream to *GCN4* promotor, the β-galactosidase activity provides indirect evidence of the *GCN4* translation.

The assay was carried out under three conditions: no stress, with amino acid limitation and with 75mM acetic acid stress. Cells were grown in presence of 2% galactose for induction of the AtGCN1c construct and GCN4-LacZ reporter. The results of the experiments are shown in Fig 4.12.. The introduction of different constructs altered the basal values of β-galactosidase activity, so the data in galactose medium were normalized with regard to those in glucose medium for every construct, and the relative behavior of every construct will be discussed separately. The experimental data indicated the overexpression of AtGCN1c in yeast reduced translation of GCN4 under the two stressed conditions assayed as compared to the basal expression under no stress conditions. This could be because AtGCN1c sequesters native yGCN2, inhibits elF2α phosphorylation and therefore the translation of *GCN4* mRNA is compromised.

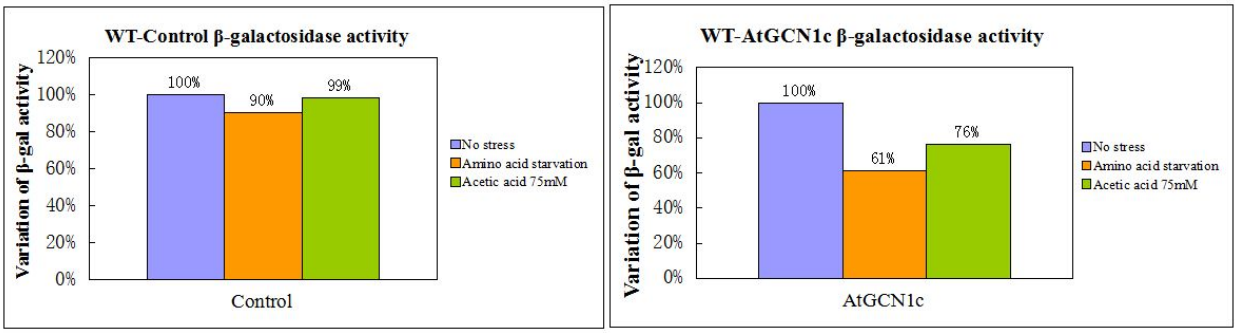

Figure 4.12. β-galactosidase enzyme activity of WT yeast strain overexpressing AtGCN1c under different stress conditions. Blue: Galactose SCD-Leu-Ura medium, no stress applied; Orange: Galactose SD-Leu-Ura medium, amino acid deprivation; Green: Galactose SCD-Leu-Ura medium with 75mM of acetic acid, acid stress.

4.3 AT3G60300

4.3.1 Experimental design and plasmid construction

Cloning of *AT3G60300* and the subsequently cells transformation procedure was the same with *GCN1*. *AT3G60300* was extracted using the previously synthesized cDNA as the DNA template. The PCR product size (1150 bp) was verified and it was cloned into pENTR™/D-TOPO®. Positive colonies were selected by kanamycin. The next step was transferring *AT3G60300* into the destination vector pAG425GAL-ccdB-HA by a LR recombination reaction The LR reaction product was transformed into *E. Coli* DH5α competent cells and those had successfully Incorporated the plasmid were selected by ampicillin and chloramphenicol. DH5α colonies successfully transformed with the plasmid were identified in the PCR colony screening.

Unfortunately, sanger sequencing indicated *AT3G60300* was not properly cloned in pENTR™/D-TOPO® and pAG425GAL-ccdB-HA. The orientation of the gene inserted is the opposite and therefore the protein could not be expressed. New primers should be designed to restart the experiment.

No further operation was carried out with this gene.

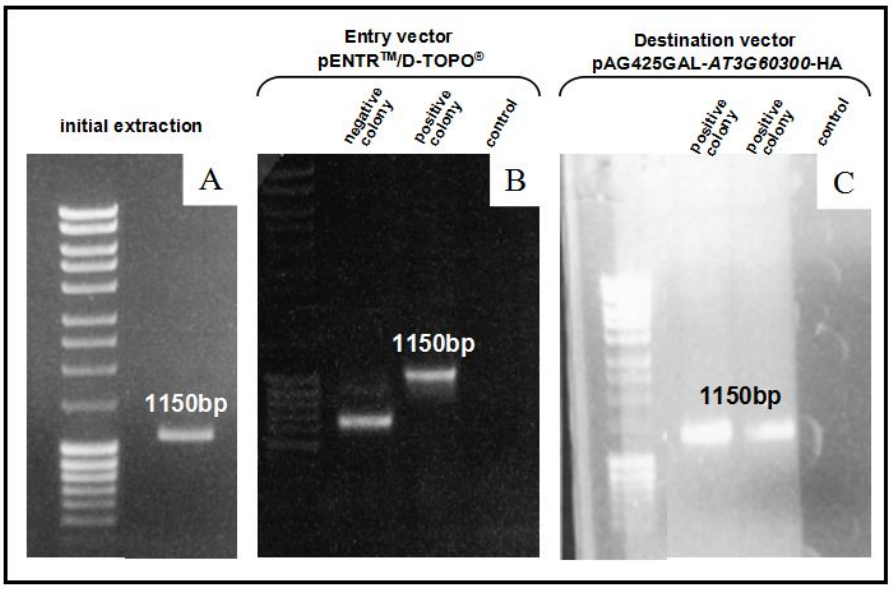

Figure 4.13. Gel electrophoresis of the PCR amplification of *AT3G60300* with gene-specific primers. A) *AT3G60300* was extracted by means of PCR amplification from plant cDNA. B) Amplification of *AT3G60300* inserted in pENTR™/D-TOPO® and screening for positive DH5α colonies. C) Amplification of *AT3G60300* inserted in pAG425GAL-*AT3G60300*-HA, the positive control correspond to the pENTR™/D-TOPO® plasmid extraction before LR recombination.

4.4 AT1G51730

4.4.1 Experimental design and plasmid construction

The *AT1G51730* cloning and subsequently cells transformation procedure was the same with GCN1. *AT1G51730* was extracted using the previously synthesized cDNA as the DNA template. After check the PCR product size (760 bp) and performed the PCR product purification. *AT1G51730* was cloned into pENTR™/D-TOPO® and positive colonies were selected by kanamycin.

The next step was the transferring of *AT1G51730* from the entry vector pENTR™/D-TOPO® to the destination vector pAG425GAL-ccdB-HA was done by means of a LR recombination reaction. The LR reaction product was then transformed into *E. Coli* DH5α competent cells, cells with the plasmid incorporated was selected by ampicillin and chloramphenicol. Colony PCR screening and sanger sequencing were also performed in order to verify the construction integrity. The result indicated pAG425GAL-*AT1G51730*-HA plasmid is successfully generated, having in its C-terminus three HA tag proteins. The plasmid was ready to be introduced into yeast cells for further manipulations (Fig 4.14).

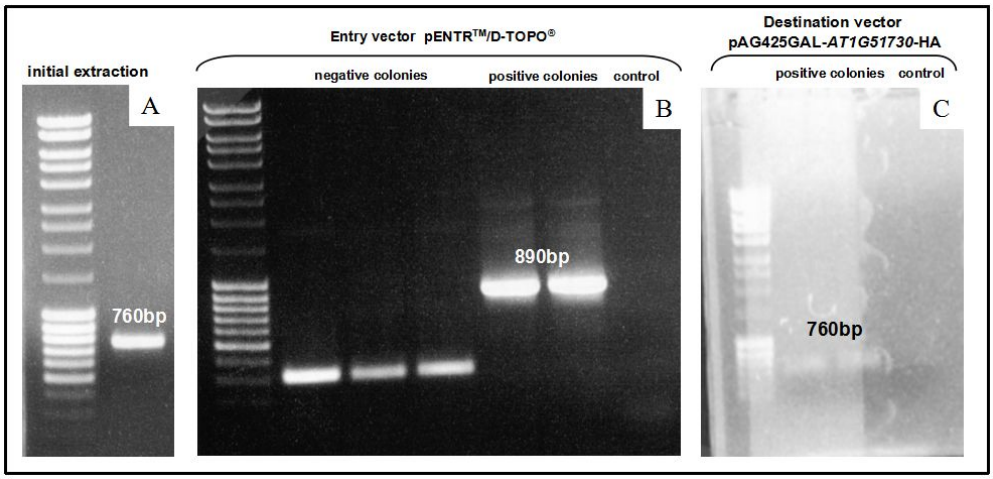

Figure 4.14. Gel electrophoresis of the PCR amplification of *AT1G51730*. A) *AT31G51730* was extracted by means of PCR amplification from plant cDNA. B) Amplification of *AT31G51730* inserted in pENTR™/D-TOPO® and screening for positive DH5α colonies using vector-based forward primer (M13) and gene-specific reward primers. C) Amplification of *AT31G51730* inserted in pAG425GAL-*AT31G51730*-HA, the positive control correspond to the pENTR™/D-TOPO® plasmid extraction before LR recombination.

WT yeast cells were co-transformed with the plasmid construction pAG425GAL-*AT1G51730*-HA. This strain already have a plasmid with a GCN4-LacZ reporter, which was used in the β-galactosidase assay to quantified the translation of GCN4.

pAG425GAL-AT1G51730-HA

ACAATCCCAACTTTGTACAAAAAAGCAGGCTCCGCGGCCGCCCCCTTCACCATGACGGAGTATAAGCAGGAGCAGGAAATGG
AGATTGAAGCTCTTTGAAGCTATACTTATGGATGAGTTTAAAGAAATTCATTCTAGTGAAAGTGGGCTTAATACTTCGAATCGAT
GCTTTCAGATTACAGTGACTCCTCAGGATGATGAACTGGAGGAATTAGCAATCCCACCAGTTCAGCTGGCTTTGGTTTTCTCGC
ACACAGAAAATTACCCGGACGAGGCTCCGCTTTTGGATGTTAAAAGTATTCGAGGAATCCATGTTAGTGACCTCACCATCTTGA
AAGAGAAGCTTGAACAAGAGGCATCTGAAAATCTAGGTATGGCCATGATCTATACACTGGTCTCATCAGCAAAGGACTGGTTGT
CTGAACACTATGGGCAAGATGATGCAGCTGAATTTGCCGGAGTAGAAGCTGCCAAAGAGGATGAGGTAATTGTCCCTCATGGA
GAACCTGTTACGCTAGAGACATTTTTGGCGTGGAGAGAGAGATATGAGGCAGAGCTTGCACTTGAGCGAGCCAAGTTGATGCC
GGAGTCTGCTCTTACAGCACCTAAGGAGAAGAAGCTTACAGGAAGACAGTGGTTCGAAAGTGGGAGAGGGAGAGGAACGGC
GGTCATTGCTGATGAGGAAGATGAGGAGGAAGATGAGGAAGACATCGACTTTGAAGACGAAGACTTTGAAGATGACGAAGAA
GACATGCTTGAGCACTATTTGGCGGAGAAATCTGATTCTTCCGCTCCCCCAACAAGGACAAAGGGTGGGCGCGCCGACCCAGC
TTTCTTGTACAAAGTGGTGATGGGCTGCAGGAATTCGATATCAAGCTTAGGTGGAATGTACCCATACGATGTTCCTGACTATGCG
GGCTATCCCTATGACGTCCCGGACTATGCAGGATCCTATCCATATGACGTTCCAGATTACGCTGCTCAGTGCTAACTCGAGGCT

Figure 4.15. Sanger sequencing reads for pAG425GAL-AT1G51730-HA.
Sequence underlined: Purple = sequence for TOPO® cloning; Green = Initial codon; Blue = Insert *AT1G51730* (completed); Orange = three HA tags; Red = Stop codon.

4.3.2. AT1G51730 expression assay

For pAG425GAL-*AT1G51730*-HA, only the wild-type strain was used. The WT yeast strain containing a GCN4-LacZ reporter was transformed with pAG425GAL-*AT1G51730*-HA, on the one hand, and with the empty plasmid pAG425GAL-ccdB-HA, on the other hand.

The western blot was carried out in the same way as shown in 4.2.2 : Induction with 2% galactose during 4 hours, protein extraction and immunoblotting. The results shown the WT strain expressed

AT1G51730 properly. AT1G51730 plus 3 HA tags gave rise to a 34kDa band, while the control strain show no band with this molecular weight (Fig 4.16).

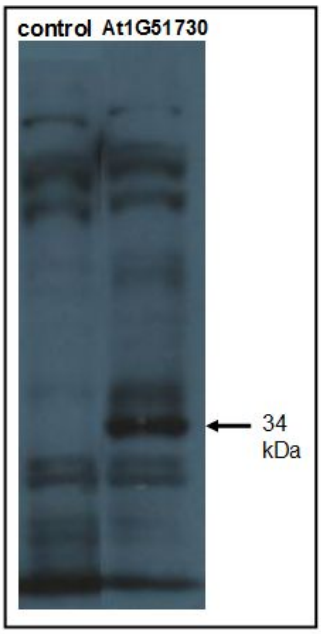

Figure 4.16. Immunodetection with anti-HA antibody for overexpression of AT1G51730 cloned in WT yeast strain. Sample were treated with 2% galactose during 4 hours. The WT strains were transformed with pAG425GAL-*AT1G51730*-HA while the control strain was transformed with pAG425GAL-*ccdB*-HA, without insert. Only the one with AT1G51730 insert cloned shown a 34kDa band corresponded to the overexpressed protein.

4.3.3 WT-AT1G51730 liquid medium assay

As mentioned before, the YIH1/IMPACT-like protein AT1G51730 shared the RWD domain with homologs of GCN2 and it was believed that AT1G51730 could compete with yGCN2 for yGCN1 binding and in this way inhibit GCN2 activation. ,

To verify the hypothesis, the same liquid medium assay was carried out for the WT strain transformed with pAG425GAL-*AT1G51730*-HA. Two different stresses were also applied to trigger the GCN2 activation: amino acid starvation (SD medium comparing with SCD) and acetic acid stress (75mM and 100mM comparing with 0mM). When At*1G51730* is overexpressed in presence of galactose, it would bind to native yGCN1 and inhibit its function. This may lead to the better growth of the WT yeast with pAG425GAL-*AT1G51730*-HA comparing to the one transformed with the empty plasmid pAG425GAL-ccdB-HA.

Again, the results shown under both amino acid deprivation (Fig 4.17) and acetic acid stress (Fig 4.18), the cell growth of the control WT strain and WT strain overexpressing AT1G51730 was the same. Even though there are evidence from the previous western blot assay that the plasmid construction was correctly generated and the AT1G51730 was successfully overexpressed in yeast. Overexpression of AT1G51730 was not able to improve the cell growth.

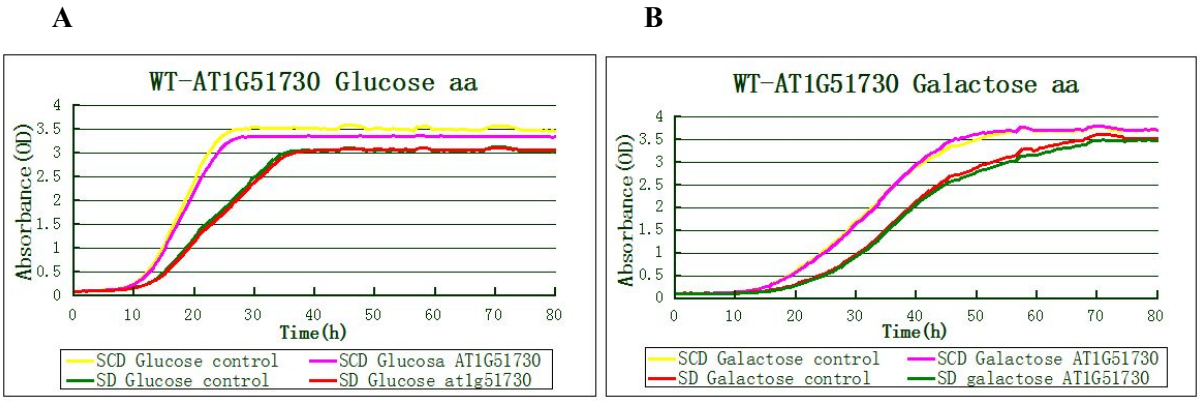

Figure 4.17. Growth curves of the control WT strain and WT overexpressing AT1G51730 under amino acid starvation. Both strains were grown in Glucose SCD-Leu-Ura medium, Glucose-SD-Leu-Ura medium (A) and Galactose SCD-Leu-Ura medium, Galactose SD-Leu-Ura medium(B). All samples were treated with 2% galactose before being analyzed in the Bioscreen reader.

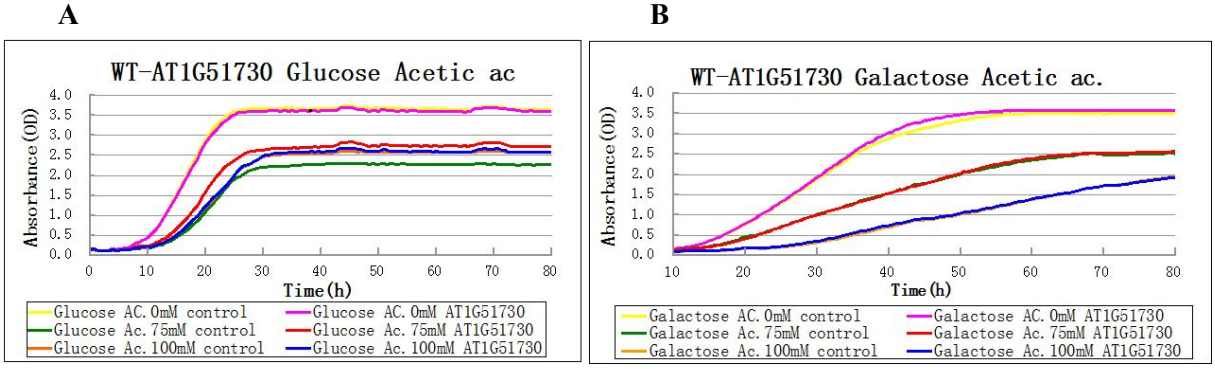

Figure 4.18. Growth curves of the control WT strain and WT overexpressing AT1G51730 under acetic acid stress (0mM, 75mM and 100mM). Bothe strains were grown separately in Glucose SCD-Leu-Ura medium(A) and Galactose SCD-Leu-Ura medium(B). All samples were treated with 2% galactose before being analyzed in the Bioscreen reader.

4.3.4 AT1G51730 β-galactosidase assay

Since no phenotypic differences were observed between the control WT strain and WT strain overexpressing AT1G51730. Another similar β-galactosidase assay was performed under three different stress conditions to quantified the translation of *GCN4* mRNA.

The experimental data were treated in the same way as explained in 4.2.5. The results indicated that β-galactosidase activity also decreased significantly when AT1G51730 is overexpressed in yeast cells under stress conditions comparing to the basal levels (Fig 4.19). The strong decrease of *GCN4* mRNA translation gives evidence that AT1G51730 might bind to yGCN1 thorough its RWD domain and might inhibit native yGCN2 by competition under stress. .

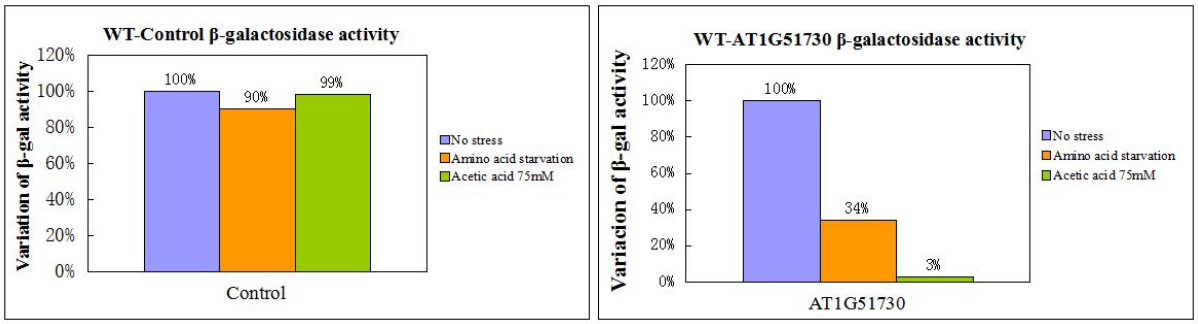

Figure 4.19. β-galactosidase enzyme activity of WT yeast strain overexpressing AT1G51730 under different stress conditions. Blue: Galactose SCD-Leu-Ura medium, no stress applied; Orange: Galactose SD-Leu-Ura medium, amino acid deprivation; Green: Galactose SCD-Leu-Ura medium with 75mM of acetic acid, acid stress.

5. Discussion

The initiation of translation is the main target of the translational regulation in response to abiotic stresses both in plants and in other eukaryotes. In yeast and mammals, elF2α phosphorylation inhibits the global protein synthesis and stimulates the selective translation of some mRNAs such as *GCN4* or *ATF4* . While mammals use four different eIF2α kinases, *Arabidopsis thaliana* has a sole GCN2-like eIF2α kinase as in yeast. AtGCN2 is structurally and functionally similar to its yeast and mammalian homologs and its activity is conserved on both eIF2α homologs.

In amino acid-deprived cells, $tRNA^{deacyl}$ accumulation triggers GCN2 activation and GCN1 is absolutely necessary for GCN2 to sense the stress signal. GCN1 binds to GCN2 through the RWD domain in GCN2. The C-terminus or region D in GCN1 comprises the keys amino acids residues for GCN1-GCN2 interaction in yeast. In *A. thaliana*, the only protein with this conserved sequence for GCN2 binding was *AT1G64790* or *ILITYHIA (AtGCN1).* Previous studies in the laboratory demonstrated *gcn1Δ* in *A. thaliana* can not phosphorylate elF2α, giving evidence that binding of AtGCN1 to AtGCN2 is essential for its activation, as in yeast and mammals. In this work, the C-terminus of AtGCN1 was overexpressed in two strains of yeast. In the $GCN2^c$ mutant strain and under amino acid starvation, overexpression of the truncated protein AtGCN1 partially suppressed the constitutively active yGCN2 and restored the defected cell growth. In the WT strain, neither under amino acid deprivation nor acetic acid stress, overexpression of the truncated AtGCN1 was not able to improve the cell growth. AtGCN1 was successfully expressed in both strain, but the interaction between AtGCN1 and yGCN2 was not strong enough to impaired yGCN2 function and restored the defected cell growth. However, although no significant phenotypic difference was observed in the WT strain, the results in the β-galactosidase activity assay indicated that when the truncated AtGCN1 was overexpressed, translation of *GCN4* mRNA was also decreased, giving evidences that AtGCN1 can somehow reduced yGCN2 activity.

On the other hand, two YIH1/IMPACT-like proteins were found in *Arabidopsis*: AT1G51730 and AT3G60300. These protein have the RWD domain and can interact with GCN1 and impair GCN2 function. In this work, the same experiments were carried out with AT1G51730 in the WT strain and the results were similar: Overexpression of AT1G51730 cannot restore the defected cell growth caused by GCN2 activation under stress conditions, although the translation of *GCN4* mRNA did decreased. Interaction between AT1G51730 and yGCN1 did lower the yGCN2 activity at the molecular level but it was not enough to produce a significant improvement in the dominant negative Gcn^- phenotype.

The inhibition of the general mRNA translation and the selective translation of certain mRNAs are crucial in the adaptation process to different abiotic stress for eukaryotic organisms. The translational regulation under stress conditions is not yet welly studied in plants. However, since the mechanism of translation and the translation factors involved in this process are very similar, the regulation of protein synthesis may also plays an important role in plants in response to different stresses. This work gives some clues about whether the mechanism for stress adaptation by elF2α phosphorylation is conserved in plants.

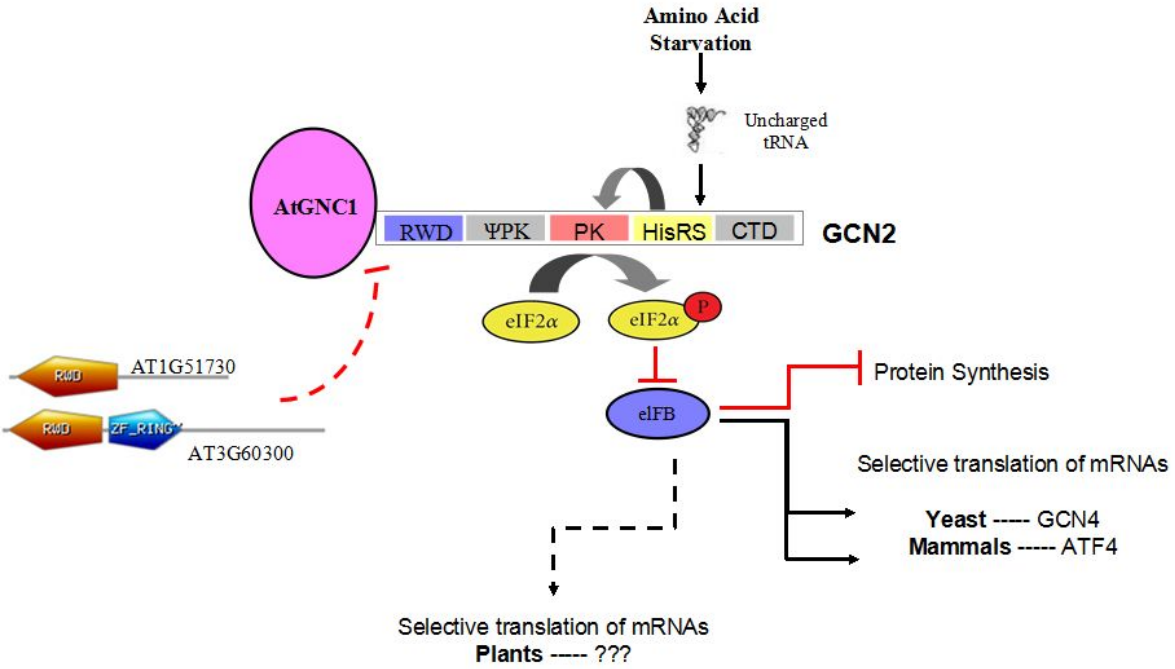

Figure 5.1. Regulation of translational initiation factors under abiotic stress conditions in plants and other eukaryotes. Stresses as amino acid deprivation leads to accumulation of $tRNA^{deacyl}$ in cell and activates GCN2 in present of GCN1. The catalytic domain in GCN2 phosphorylates elF2α and inhibit elFB. Inactivation of elFB leads to inhibition of the global protein synthesis and stimulation of the selective translation of mRNAs, which are still unknown in plants. AT1G51730 and AT3G60300 may compete for GCN1 binding through their RWD domain and impair GCN2 activation and the subsequent GAAC response.

The experimental data shown AtGCN1 and AtGCN2 exhibits a high primary sequence homology to their yeast and mammalian counterparts. The GCN1-GCN2 interaction mode can be also identified in plants and AtGCN1 may be needed for AtGCN2 activation . At the moment, it has been demonstrated that phosphorylation of elF2α leads to inhibition of protein synthesis during amino acid deprivation in *A. Thaliana*, but it is not known if this phosphorylation also stimulate translation of specific mRNAs as reported for other organisms, and no homolog to GCN4 or ATF4 transpirational factors has been identified (Muñoz and Castellano., 2011). Nevertheless, in this work, overexpression of AtGCN1c in yeast induced variation of the *GCN4* mRNA translation. These results help to demonstrate that the phosphorylation of elF2α is conserved in plants as a strategy for stress adaptation, despite of the divergent downstream reactions. Moreover, both AT1G51730 and AT3G60300 contain the structures necessary for association with GCN1. Indeed, overexpressed AT1G51730 seemed to reduce *GNC4* translation levels in yeast. Could they be negative regulators of the GAAC response under normal conditions? Certainly more studies must be carried out to shed light on this question.

6. Conclusions

- ✓ AT1G64790 or AtGCN1 is the yeast GCN1 homolog in plant, the only protein in *A.thaliana* with the keys amino acids for GCN2 binding.

- ✓ The two YIH1/IMPACT-like proteins found in *Arabidopsis* were AT1G51730 and AT3G60300. Both share the RWD domain or the GCN1-interacting domain with homologs of GCN2 and IMPACT.

- ✓ Overexpression of the AtGCN1 C-terminal segment (AtGCN1c) in constitutively active GCN2 mutant strain ($Gcn2^{c}$), restore the defected cell growth characteristic of this strain under amino acid starvation conditions.

- ✓ Overexpression of the AtGCN1 C-terminal segment (AtGCN1c) in wild-type yeast cells was not sufficient to produce the dominant negative Gcn^{-} phenotype. Neither under amino acid starvation nor acetic acid stress.

- ✓ Overexpression of AtGCN1c and AT1G51730 in wild-type yeast cells reduces GCN4 expression under amino acid starvation and acetic acid stress condition putatively by dampening the activation of the native yGCN2.

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