
# *SnRK1β1 interacting proteins: a new approach to the SnRK1 function*

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# Resumo

O *stress* biótico ou abiótico e a falta de nutrientes, têm um efeito negativo muito grande no normal desenvolvimento das plantas. Desta forma, a planta precisa de alterar o seu metabolismo, para produzir energia, através de vias metabólicas alternativas para reestabelecer a homeostasia e sobreviver. O complexo SnRK1 *(SNF1-related protein kinase)* tem um papel muito importante na coordenação desta resposta. Sob condições de *stress* energético, o complexo SnRK1 é ativado e leva a alteração na expressão de mais de mil genes que permitem, então, à planta alcançar a homeostasia e ter uma resposta adaptativa mais específica.

Apesar da importância que a SnRK1 tem no crescimento e desenvolvimento da planta, o nosso conhecimento atual sobre esta via de sinalização é limitado. O complexo SnRK1 é ortólogo de SNF1, de leveduras, e de AMPK presente em mamíferos, e, portanto, a caracterização do complexo SnRK1 em plantas, baseou-se no conhecimento que já se tinha destes outros complexos. Contudo, ainda que muito valiosa e bem-sucedida, esta abordagem é limitada no que diz respeito à descoberta de novas interações únicas na planta. A fim de identificar alvos a jusante de SnRK1, foi realizado um rastreio de dois híbridos em levedura (Y2H), utilizando SnRK1β1.

Este rastreio foi realizado usando uma biblioteca de cDNA de *Arabidopsis* (Clontech) e permitiu a identificação de 64 proteínas que supostamente interagem com esta subunidade β1. Mais de metade das proteínas identificadas estão relacionadas com o metabolismo, estando de acordo com a função de SnRK1 na resposta metabólica à falta de energia. Curiosamente, algumas destas proteínas estão envolvidas em processos de *splicing*, metabolismo do azoto, bem como fatores de transcrição da família das KNATs.

**Palavras-chave:** Sinalização do *stress*; *Arabidopsis thaliana;* Complexo SnRK1; SnRK1β1.

# Abstract

Biotic or abiotic stresses, and nutrient starvation have a profound negative effect in the normal plant development. Thus, the plant needs to change its metabolism to produce energy through metabolic alternative pathways in order to re-establish energy homeostasis and to survive. SnRK1 (SNF1-related protein kinase) complex plays an important role in the coordination of this response. Under energy stress conditions, SnRK1 complex is activated and trigger changes in the expression of over one thousand genes that allow the re-establishment of homeostasis and the mounting of a more specific adaptive response.

Despite the importance of SnRK1s for plant growth and development, our current knowledge on this signalling pathway is limited. SnRK1s are the orthologs of the yeast SNF1 and the mammalian AMPK, and so far most of the characterization of the SnRK1 system has relied on knowledge from these systems. However, although very valuable and successful, this approach is limited in its ability to uncover novel interactions unique to plants. In order to identify downstream targets of the SnRK1, we performed an yeast two hybrid screen using SnRK1β1 as bait.

The Yeast-two hybrid screen assay was done using an *Arabidopsis* cDNA library (Clontech) and allowed the identification of 64 proteins that putatively interact with the β1-subunit. More than half of the proteins identified are functionally related to metabolism, in line with the important role of SnRK1 in the metabolic response to energy deficit. Interestingly, some of the novel interactors identified include proteins involved in the splicing process, nitrogen metabolism and transcription factors of the KNAT family.

**Keywords:** Stress signalling; *Arabidopsis thaliana*; SnRK1 complex; SnRK1β1; Proteins

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# Abbreviations

**AD –** Plasmid encoding the yeast Gal4 activation domain

**Ade (A)/ His (H)/ Leu (L)/Trp (W) –** Adenine/ Histidine/ Leucine/ Tryptophan

**ADP –** Adenosine diphosphate

**AMP –** Adenosine monophosphate

**Amp** – Ampicillin

**ATP –** Adenosine triphosphate

**cDNA –** Complementary DNA

**cfu** – Colony-Forming Unit

**DNA-BD** - Plasmid encoding the Gal4 DNA-binding domain

**EDTA –** Ethylenediaminetetraacetic Acid

**LiAc –** Lithium acetate

**mM/mL –** millimolar/milliliter

**PCR –** Polymerase Chain Reaction

**PEG –** Polyethylene glycol

**SD** - Minimal, synthetically defined medium for yeast; is comprised of a nitrogen base, a carbon source (glucose unless stated otherwise), and a DO supplement

**SD -2/SD -4** - Minimal, synthetically defined medium for yeast without Leucine (L) and Tryptophan (W)/ without leucine (L), tryptophan (W), Adenine (A) and histidine (H)

**TE buffer –** Tris+EDTA

**Tris –** Tris(Hydroxymethyl)aminomethane

**WT** – Wild type

**Y2H** – Yeast Two-Hybrid System

**YPD** - A blend of yeast extract, peptone, and dextrose in optimal proportions for growth of most strains of *S. cerevisiae*

**YPDA** - YPD medium supplemented with adenine hemisulfate (1X concentration = 120 μg/ml)

**μM/μL –** micromolar/microliter

# Introduction

# 1. Introduction

## 1.1. Stress signalling

The ability of an organism to respond to adverse environmental conditions is critical for its survival. Energy supplies varies over the time and it was necessary to develop sophisticated mechanisms that perceive fluctuation in nutrient availability and are able to maintain the energy balance at cellular and organism levels. These mechanisms, which respond to energy depletion, are conserved in all eukaryotes and due to its importance they have been extensively studied (Hardie *et al.*, 1998; Xiong *et al.*, 2002; Halford *et al.*, 2003; Hardie *et al.*, 2012; Cabello *et al.*, 2014; Crozet *et al.*, 2014). Specifically in plants, energy deprivation is caused not only by nutrient depletion but also by biotic and abiotic stresses. Indeed, severe environmental factors such as low temperature, drought, high salinity, darkness and pathogens cause biochemical, molecular and physiological changes in plants that might compromise photosynthesis and/or respiration leading, in this way, to energy stress. Drought and salinity are the two major adverse environmental factors because they prevent plants from realizing their full genetic potential and reduce crop productivity being responsible, in severe conditions, for up to 65% reduction in yield, much higher than the losses caused by diseases and insects that, while devastating to individual farmers, globally are generally less than 10% (Serrano *et al.*, 1999; Zhu, 2002; Tuteja, 2007).

Environmental stress and the consequent energy deficit cause cessation of growth, activation of catabolic pathways and a decline of biosynthetic activity as well as the activation of autophagy in order to re-establishment the cellular homeostasis (Baena-Gonzalez and Sheen, 2008; Akpinar *et al.*, 2012).

## 1.2. AMPK/SNF1/SnRK1 Complex

Despite the crucial function of other kinases, the AMPK/SNF1/SnRK1 family of protein kinases appears to be the central regulator of the stress response playing an important role as metabolic sensors. These kinases belong to a highly conserved protein kinase family with a N-terminal kinase domain being found throughout Eukaryotes, as represented below in figure 2, among which are the insects, roundworms, mammals, fungi and plants (Hardie *et al.*, 1998; Hardie, 2007; Polge and Thomas, 2007; Hedbacker and Carlson, 2008; Baena-Gonzalez, 2010; Ghillebert *et al.*, 2011). The first experimental evidences of the presence of AMP-activated protein kinase in rat was observed in 1973 by two independent studies (Beg *et al.*, 1973; Carlson and Kim, 1973). This protein was allosterically activated by AMP levels and so it was called AMP-activated protein kinase (AMPK) (Hardie, 2007; Ghillebert *et al.*, 2011). Sucrose non-fermenting 1 (SNF1) is an ortholog of AMPK and was identified in *Saccharomyces cerevisiae* in 1981 when the *snf1* mutation was discovered among other mutants that were unable to utilize sucrose (Carlson *et al.*, 1981). Later, sequence similarity has revealed the existence in plants of three sub-families related to AMPK and SNF1 i.e. SNF1-related protein kinases (Halford and Hardie, 1998; Crozet *et al.*, 2014).

The AMPK/SNF1/SnRK1 has been found in all eukaryotes as heterotrimeric complexes comprising one catalytic subunit (α-subunit) and two regulatory subunits (β- and γ-subunits) (figure 1b) (Bouly *et al.*, 1999; Polge and Thomas, 2007; Baena-Gonzalez and Sheen, 2008; Baena-Gonzalez, 2010; Hardie *et al.*, 2012; Crozet *et al.*, 2014). The response of this complex to a specific stimuli depends on the tissue, the development stage and on the precise subunit composition of the complex (Ramon *et al.*, 2013; Nietzsche *et al.*, 2014).

AMPK/SNF1/SnRK1 catalytic α-subunit has a conventional Serine (Ser)/Threonine (Thr) kinase domain that is activated by phosphorylation in a specific conserved Thr residue, present in the activation loop, by upstream kinases (Hardie *et al.*, 2012). The specific residue that is phosphorylated depends on the species, for example in the case of *Arabidopsis* it is $Thr^{175}$ (α1) or $Thr^{176}$ (α2) (Sugden *et al.*, 1999a; Shen *et al.*, 2009; Crozet *et al.*, 2010), in AMPK is the $Thr^{172}$ (in human α1) (Hawley *et al.*, 1996) and $Thr^{210}$ in case of yeast SNF1 (McCartney and Schmidt, 2001). In animals and yeast, the C-terminal region of α-subunit contains an auto-inhibitory domain/sequence (AID or AIS) which inhibits kinase activity (figure 1a) (Hardie *et al.*, 2012; Crozet *et al.*, 2014). Following this region, there is an α-

subunit carboxy-terminal domain (α-CTD) where the β-subunit will bind (figure 1a) (Hardie *et al.*, 2012; Crozet *et al.*, 2014). In plants the AID region has not the same role as in animals and yeasts and it is called Ubiquitin-Associated Domain (UBA) (figure 1a) because it seems to mediate the interaction with ubiquitinated proteins (Crozet *et al.*, 2014). With a function similar to animals and yeasts α-CTD, SnRK1α (or AKINα) contains a Kinase Associated 1 (KA1) domain responsible for the interaction with other subunits and the upstream phosphatases (figure 1a) (Rodrigues *et al.*, 2013; Crozet *et al.*, 2014)

The typical β-subunits (AMPKβ1/β2, SNFsip1/sip2/Gal83 and SnRK1β1/β2) play an essential role in specificity and the recognition by the kinase complex of its targets and they are also responsible for the subcellular localization of the complex which is dependent on the presence of N-terminal myristoylation (N-MYR) of the β1- and β2-subunits (figure 1a) (Warden *et al.*, 2001; Lin *et al.*, 2003; Polekhina *et al.*, 2005; Li *et al.*, 2009; Avila *et al.*, 2012; Liang *et al.*, 2015). N-MYR of the SnRK1β1 appears to negatively regulate nuclear SnRK1 activity by sequestering the complex at the plasma membrane (Pierre *et al.*, 2007). N-MYR is required for the regulation of SnRK1 and N-MYR defect of the SnRK1βs prevents shoot apical meristem (SAM) differentiation and leads to development arrest. (Pierre *et al.*, 2007; Traverso *et al.*, 2008). Accumulation of StubGal83 mRNA (potato gene that encodes β-subunit) as well as of SnRK1β1 (*Arabidopsis* β-subunit) is induced by darkness and this effect is reversed by a light treatment. Moreover, StubGal83 is involved in root and tuber development (Lovas *et al.*, 2003). SnRK1β1 seems to modulate leaf and cotyledon shape in response to metabolic sugars and specifically participates in regulation of Nitrate reductase (NR) and the interaction between SnRK1.1/1.2 (α-subunit) and NR2 (one of the NR genes) (Schmidt and McCartney, 2000; Polge *et al.*, 2008; Li *et al.*, 2009). The β-subunits also possess a carbohydrate-binding module (CBM) or Glycogen-binding domain (GBD) (figure 1a) inserted in KIS (Kinase Interaction Sequence) region. In AMPK, GBD serves to bind glycogen, however, in SNF1, GBD motif seems to have not the same function because blocking glycogen synthesis has no impact in SNF1 activity and deletion of the GBD motif in Gal83 (one of the SNF1 β-subunits) increased the activity of SNF1 (Avila-Castaneda *et al.*, 2014; Emanuelle *et al.*, 2015). In plants, the function of GBD motif of SnRK1 β-subunits still remains unclear (Emanuelle *et al.*, 2015). Another important function of the β-subunits is to act as a scaffold keeping the α and γ together through the Association

with the SNF1 Complex (ASC) domain (Avila-Castaneda *et al.*, 2014) also called Subunit Binding Sequence (SBS) (figure 1a and b) (Polekhina *et al.*, 2005).

The γ-type subunit is composed by four tandem cystathionine β–synthase (CBS) motifs that function as dimers forming two domains called Bateman domain 1 and 2 (figure 1a) that can bind the regulatory adenine nucleotides (AMP/ADP/ATP) providing the primary means of AMPK catalytic regulation. AMP is the only adenylate that directly activates AMPK (Oakhill *et al.*, 2011; Hardie *et al.*, 2012; Emanuelle *et al.*, 2015). However, this allosterically effect of the adenine nucleotides is not observed in the case of yeast SNF1 (Mayer *et al.*, 2011) and plant SnRK1 (Shen *et al.*, 2009). Plant SnRK1γ complements yeast snf4Δ (Bradford *et al.*, 2003; Rosnoblet *et al.*, 2007). In addition, the γ-subunit of *Medicago truncatula* (MtSNF4b) seems to play an important role in seed longevity and in fruit ripening (Rosnoblet *et al.*, 2007; Bolingue *et al.*, 2010).

Beyond these subunits, which are generally conserved throughout eukaryotes, there are two other subunits (β3 and βγ) that are atypical and are only present in plants (figure 1a) (Polge and Thomas, 2007; Avila-Castaneda *et al.*, 2014; Crozet *et al.*, 2014). SnRK1βγ is able to suppress yeast snf4Δ mutation and it is constitutively expressed in all plants and seems to be more important than SnRK1γ in the SnRK1 complex composition (Lumbreras *et al.*, 2001; Ramon *et al.*, 2013). This subunit is similar to SNF4/AMPKγ with the four tandem CBS motif but contain an extended N-terminal region similar to the KIS domain (containing the GBD region) of AMPK/SNF1 β-type (figure 1a) (Lumbreras *et al.*, 2001; Lopez-Paz *et al.*, 2009). Furthermore, according to the work done by Ramon *et al.*, (2013) *Arabidopsis* SnRK1γ is not directly involved in SnRK1 signalling since knock-out (KO) of this subunit caused no differences in transcript levels of one of the SnRK1 target genes. Whereas SnRK1βγ RNAi (RNA interference) caused significant differences in target genes expression suggesting that this subunit is needed for formation of the heterotrimeric complex of SnRK1 instead of γ-subunit (Lumbreras *et al.*, 2001; Ramon *et al.*, 2013).

The SnRK1β3 subunit presents a truncated KIS domain and no N-terminal region comprising only the C-terminal domain (ASC) which is closely related to the ASC domains present in SnRK1β1 and SnRK1β2 (figure 1a). This subunit interacts with both α- and γ-subunits and it is also capable to complement β-deficient yeast (sip1Δsip2Δgal83Δ) (Gissot *et al.*, 2004; Polge *et al.*, 2008; Ghillebert *et al.*, 2011; Emanuelle *et al.*, 2015). Additionally

SnRK1β3 transcripts were found in all the organs and at different development stages. A strong interaction is observed between SnRK1β3 and SnRK1βγ but not with SnRK1γ (Gissot *et al.*, 2004).

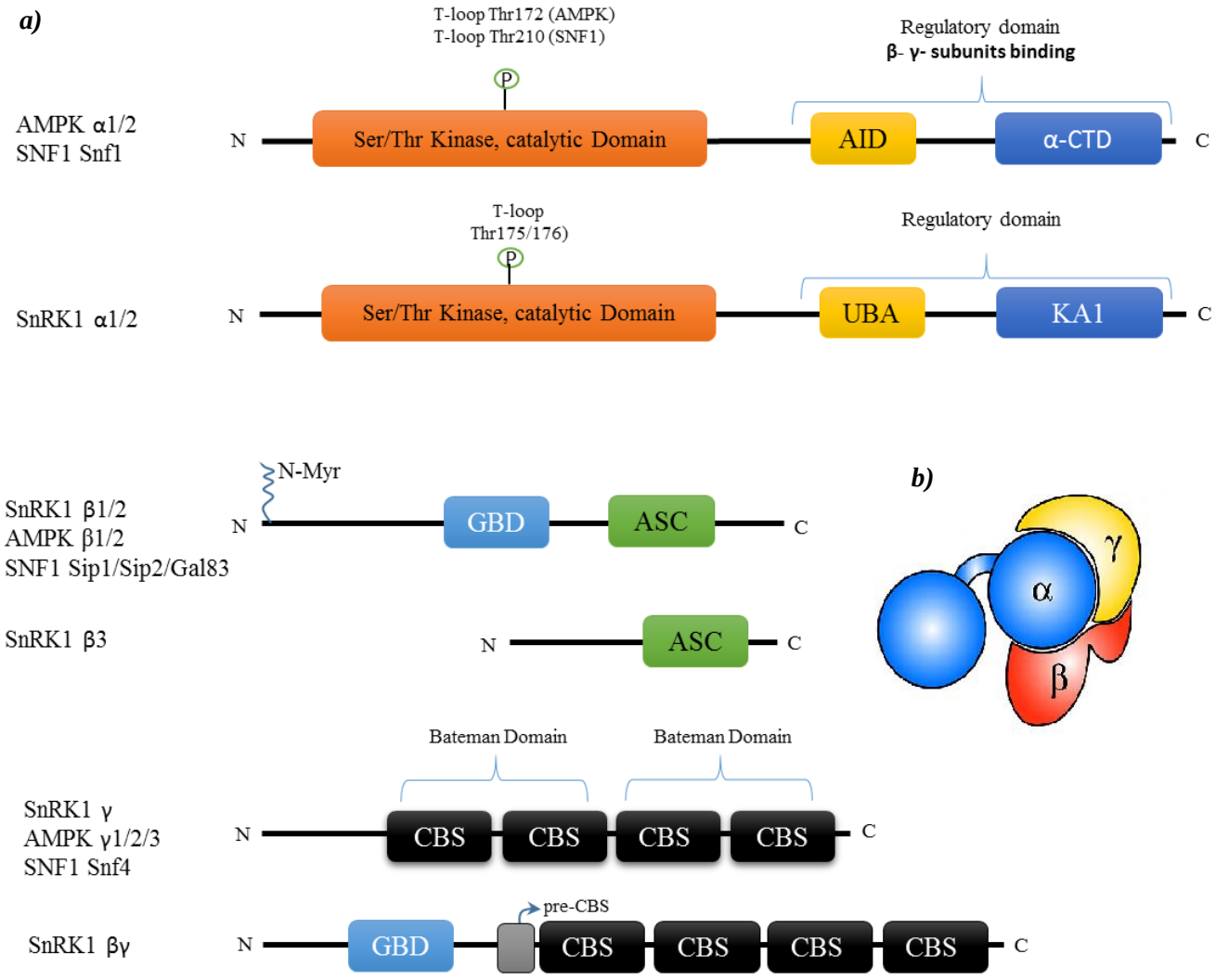


***Figure 1: SNF1/AMPK/SnRK1 subunit domain architecture and subunit domains.***

*a) The major regulatory phosphorylation site is the T–loop threonine residue in the subunit catalytic domain. The residue that is phosphorylated differs among animals, fungi and plants. UBA - ubiquitin-associated domain (just in plants); AID - Auto-inhibitory domain (present in animals and yeasts); KA1 - kinase-associated 1 domain (plants); CTD - α-subunit carboxy-terminal domain (animals and yeast); N–Myr - N–myristoylation site; CBM - carbohydrate-binding module; CBS - cystathionine β–synthase motif; The plant βγ-subunit has a pre-CBS and GBD domain similar to β-subunits.*

*b) Complex structure showing how the subunits interact with each other to form the active complex. This structure is common to the three kingdoms. (Adapted from Crozet et al., 2014; Emanuelle et al., 2015)*

### 1.2.1. Mammalian AMPK

So AMPK has been characterized as a mammalian protein kinase that is allosterically activated by AMP leading to the inactivation of the biosynthetic pathways while catabolic pathways such as fatty acid oxidation, glycolysis and autophagy are activated, providing in this manner the energy that the cells need (Bouly *et al.*, 1999; Hardie, 2007; Ghillebert *et al.*, 2011; Hardie, 2011; Oakhill *et al.*, 2011; Hardie *et al.*, 2012; Crozet *et al.*, 2014). Recent studies have shown that AMPK may be sensitive to the intracellular fatty acids availability in an independent manner of the cellular AMP levels (Clark *et al.*, 2004) and this provide an increase activity of AMPK due to increase of AMPK affinity with LKB1, an upstream kinase (Watt *et al.*, 2006). Thus fatty acids are able to allosterically activate AMPK (Clark *et al.*, 2004; Watt *et al.*, 2006). Beyond the allosteric activation, high levels of ADP:ATP ratio and cytosolic $Ca^{2+}$ are also capable to activate the kinase domain of the AMPK Complex (Ghillebert *et al.*, 2011; Hardie *et al.*, 2012). So AMPK is also indirectly activated by metabolic stresses that inhibit ATP production (hypoxia, glucose deprivation) or stimulate ATP consumption (motor proteins, ion pumps, biosynthetic pathways). Cytokines, ciliary neurotrophic factor (CNTF) and certain drugs are also responsible for AMPK activation (Hardie, 2007). Activation of AMPK promotes phosphorylation of metabolic enzymes, transcription factors and co-activators followed by regulation of gene expression (Hardie, 2007).

The activation of the complex is performed in three steps: first, AMP binds to γ-subunit stimulating α-subunit. Next, this binding promotes the phosphorylation of the Thr residue ($Thr^{172}$) in the catalytic subunit (Hardie, 2007). Third, the activation of the complex requires the N-terminal myristoylation of the AMPKβ that will regulates subcellular localization and kinase activity acting as a scaffold protein (Warden *et al.*, 2001; Oakhill *et al.*, 2010). The major upstream kinase is LKB1 associated to two accessory subunits, STRAD and MO25, to form the LKB1–STRAD–MO25 complex (identified genetically as a tumor suppressor), and it is responsible for the function of AMPK of inhibiting cell growth and proliferation and promote cell polarity. $Ca^{2+/}$calmodulin-activated protein kinase kinases (also known as CaMKK2) and TAK1 (transforming growth factor-β [TGFβ]-activated kinase 1) are other two upstream kinases that are able to phosphorylate AMPK, depending on cellular and activation context (Hardie, 2007; Crozet *et al.*, 2010; Hardie *et al.*, 2012; Liang and Mills, 2013; Crozet *et al.*, 2014). CaMKKs, appears to act in cells with an increased

levels of cytosolic $Ca^{2+}$ and to be able to activate AMPK even in the absence of high AMP levels (Hardie *et al.*, 2012).

More importantly, it is well known the effects of AMPK on metabolism but it may also regulate energy levels by other pathways. Two of those, are regulation of cell cycle and modulation of the nervous system (Hardie *et al.*, 2012). The DNA replication (S phase) and mitosis (M phase) are processes that require energy to go through. The activation of AMPK in cycling cell causes arrest of cell cycle in G1 phase. This event is associated with phosphorylation of p53 (a tumor suppressor) by AMPK, in turn p53 will activate the expression of cyclin-dependent kinase inhibitor 1A (CDKN1A; also known as $p21^{WAF1}$) (Jones *et al.*, 2005; Liang *et al.*, 2007; Hardie *et al.*, 2012). This signal leads to cell survival while inhibit cell proliferation (Liang and Mills, 2013; Liang *et al.*, 2015). AMPK is also required for maintenance of nervous system (Tschape *et al.*, 2002; Ghillebert *et al.*, 2011) and in *Drosophila* a mutation in AMPKγ results in a neurodegenerative phenotype (Tschape *et al.*, 2002). Additionally, KO of AMPK in *Drosophila melanogaster* provokes damages of the nervous system and defects on epithelial integrity and cell division (Tschape *et al.*, 2002; Lee *et al.*, 2007; Spasic *et al.*, 2008).

### 1.2.2. Yeast SNF1

In yeast, the AMPK ortholog, Sucrose non-fermenting 1 (SNF1) is activated mainly by glucose absence leading to the subsequent activation of a plethora of genes allowing yeast to utilize alternative carbon sources (e.g. sucrose, glycerol or ethanol) (Carlson *et al.*, 1981; McCartney and Schmidt, 2001). Besides sugar signalling, there are many types of conditions that induce SNF1 activation such as sodium ion stress, oxidative stress, alkaline pH and inhibitors of respiratory chain (Hedbacker and Carlson, 2008; Ghillebert *et al.*, 2011). Contrary to what happens with the AMPK, SNF1 is not allosterically activated by AMP levels (Ghillebert *et al.*, 2011) while ADP appears to regulate SNF1 T-loop phosphorylation protecting the kinase from dephosphorylation (Mayer *et al.*, 2011). In particular, the β subunits of SNF1 complex exhibits also very important role as each one alone (sip1 or sip2 or gal83) is sufficient for yeast growth in media with different carbon sources like glucose, galactose, synthetic complete media with raffinose or glycerol-ethanol (Schmidt and McCartney, 2000). Additionally, the N-terminal domain of the β1- and β2-subunits confers unique cellular localization of the SNF1 kinase upon glucose depletion and specific β-subunit which

are required for substrate definition (Schmidt and McCartney, 2000; Vincent *et al.*, 2001; Hedbacker and Carlson, 2008). The γ-subunit (Snf4) have the same structure as AMPKγ (figure 1a) with four CBS domains but Snf4 has a substitution in a residue which is determinant for allosterically connection of AMP to the γ-subunits. However Snf4 is still required for the catalytic activity of the heterotrimeric kinase (Celenza and Carlson, 1984, 1989; McCartney and Schmidt, 2001). If necessary, SNF1 inactivates itself by binding of AID (snf1 regulatory domain) to the snf1 kinase domain or to the Snf4 (Lin *et al.*, 2003; Hedbacker and Carlson, 2008).

SNF1 catalytic activity is regulated by three upstream kinases: Elm1, Tos3 and Sak1 (Hong *et al.*, 2003; Nath *et al.*, 2003; Sutherland *et al.*, 2003; Hedbacker *et al.*, 2004). They exhibit overlapping functions as only the triple mutant of these kinases (elm1Δsak1Δtos3Δ) shows the same phenotype of snf1Δ (Hong *et al.*, 2003). The catalytic domain of the three yeast upstream kinases are similar to those of CaMKKs and LKB1 (Hong *et al.*, 2003). Despite all three being functionally redundant, they have different contribution to cellular regulation under different growth conditions (Kim *et al.*, 2005) as well as in its cellular localization (Hong *et al.*, 2003; Kim *et al.*, 2005). SNF1 is inactivated by dephosphorylation by the protein phosphatase type 1 GLC7 in association with the regulatory subunit REG1 (Tu and Carlson, 1995; Ludin *et al.*, 1998). Interestingly, it seems that glucose does not change Glc7-Reg1 activity, but rather indirectly controls SNF1 dephosphorylation by changing the ability of the activation to be dephosphorylated by the phosphatase (Ruiz *et al.*, 2011).

Once activated, SNF1 triggers a transcriptional reprogramming achieved by the activation of various TFs and the inactivation of others. This will allow the expression of a large set of genes (over 400 genes) including many of the glucose-repressed genes through control of the transcriptional repressor Mig1 (Treitel *et al.*, 1998). Under low glucose conditions, SNF1 phosphorylates Mig1 leading to its nuclear export (Lin *et al.*, 2003; Hedbacker and Carlson, 2008; Ghillebert *et al.*, 2011; Cho *et al.*, 2012). Other TFs are activated by the SNF1 complex like Cat8 and Sip4 which will activate gluconeogenic genes expression (Hedges *et al.*, 1995; Lesage *et al.*, 1996).

Additionally, SNF1 switch from fermentative to oxidative metabolism and gluconeogenesis (Bouly *et al.*, 1999; Polge and Thomas, 2007; Baena-Gonzalez, 2010; Hardie *et al.*, 2011; Crozet *et al.*, 2014; Emanuelle *et al.*, 2015) and is also involved in controlling the

biosynthesis of reserve carbohydrates and activates autophagy to recycle macromolecules and organelles (Ghillebert *et al.*, 2011). In addition, SNF1 has an important role in development processes such as meiosis, ageing, growth (diploid pseudohyphal growth and haploid invasive growth) and proliferation as well as mating and sporulation (Hedbacker and Carlson, 2008; Ghillebert *et al.*, 2011; Crozet *et al.*, 2014). Overexpression of Snf1 and the absence of N-myristoylation of Sip2 promote aging and this effect is unique among Snf1 β-subunits (Lin *et al.*, 2003; Crozet *et al.*, 2014). On the contrary, the absence of Snf4 extends life span (Schmidt and McCartney, 2000; Lin *et al.*, 2003).

### 1.2.3. Plant SnRK1

The large *Arabidopsis* SnRK superfamily is composed of three distinct subfamilies: SnRK1, SnRK2 and SnRK3 (figure 2) (Hrabak *et al.*, 2003; Thelander *et al.*, 2004). The SnRK2 and SnRK3 are known for their involvement in stress and abscisic acid signalling (Hrabak *et al.*, 2003). The SnRK1 subfamily comprises SnRK1.1/SnRK1.2/SnRK1.3 (also known as KIN10/KIN11/KIN12) that are the closest relatives to SNF1 and AMPK complexes (figure 2) (Baena-Gonzalez, 2010; Crozet *et al.*, 2014) and it has been studied in several plant species (besides *Arabidopsis*) such as tomato, rice, potato, tobacco, pea and barley (Li *et al.*, 2010). SnRK1.3 has a very low expression during *Arabidopsis* development as compared to the other two catalytic subunits (Fragoso *et al.*, 2009). SnRK1.1 and SnRK1.2 are 81% identical. However SnRK1 activity in plant cells is mostly held by SnRK1.1 (Jossier *et al.*, 2009), while the SnRK1.2 may plays a restricted role in regulation of metabolism and stress signalling (Williams *et al.*, 2014).

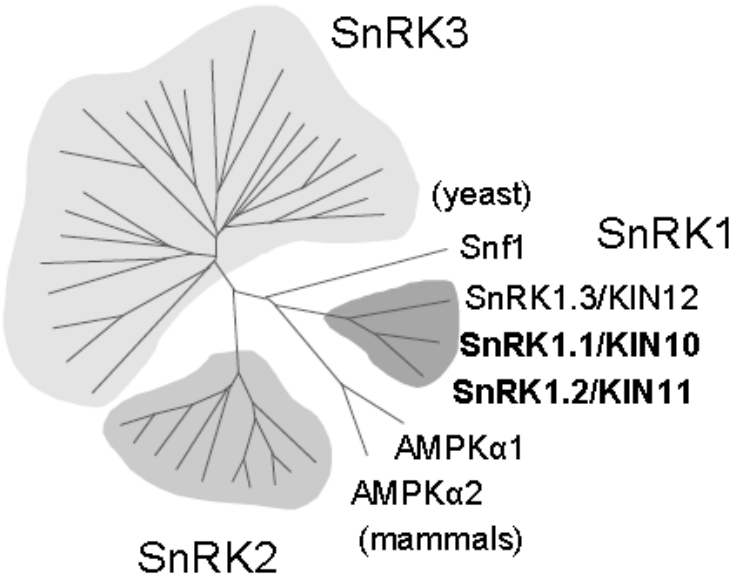


***Figure 2: The Arabidopsis SnRK superfamily comprises 3 groups.*** *The SnRK1s are most homologous to yeast Snf1 and mammalian AMPK. The other two groups are also related to stress and abscisic acid signalling pathways. (Baena-Gonzalez et al., 2007)*

SnRK1 was identified in *Arabidopsis* and in other plants and appears to have pleiotropic roles in response to hormonal, nutritional and environment stresses (figure 3) (Hardie *et al.*, 1998; Wang *et al.*, 2003; Baena-Gonzalez, 2010; Ghillebert *et al.*, 2011). SnRK1 complex is activated and required to respond to sugar depletion or to long darkness period but not by an increase of the AMP:ATP ratio, as happens with AMPK complex (Baena-Gonzalez *et al.*, 2007; Baena-Gonzalez and Sheen, 2008; Rosa *et al.*, 2009). However, the physiological concentrations of 5'-AMP are able to inhibit the T-loop dephosphorylation of the spinach SnRK1 kinase (Sugden *et al.*, 1999b). Recent studies have identified *Arabidopsis* SnRK1.1/SnRK1.2 as central integrators of the transcriptome in response to darkness events, sugar deprivation and stress conditions (figure 3) (Baena-Gonzalez *et al.*, 2007). Double SnRK1.1/SnRK1.2 deficiency in *Arabidopsis thaliana* cause the transcription of genes associated to darkness and stress signalling to be switch off, resulting in impaired starch mobilization and growth defects in plant mutants under 13h light/11h dark or constant illumination (Baena-Gonzalez *et al.*, 2007). Similar results were obtained in *Physcomitrella patens* (Thelander *et al.*, 2004). Overexpression of two SnRK1 genes of *Malus hupehensis* (MhSnRK1/2) in tomato resulted in fruit ripening 10 days earlier than WT and a higher root/shoot ratio in transgenic plants. Consistent with this observation, the starch content and soluble sugar were found to be higher in leaves and red-ripening fruit of transgenic tomato

plants than in WT (Li *et al.*, 2010; Wang *et al.*, 2012). The higher photosynthesis rate observed in tomato leaves overexpressing MhSnRK1 suggest that SnRK1 may influence photosynthesis (Wang *et al.*, 2012).

Plant senescence is also influenced by SnRK1 activity. The *Arabidopsis* SnRK1 double mutant senesced before flowering and those that bolted were not able to produce viable inflorescences and auxiliary floral meristem, showing the importance of SnRK1 in plant life and development (Thelander *et al.*, 2004; Baena-Gonzalez *et al.*, 2007). On the contrary, SnRK1.1 overexpression cause starvation tolerance and lifespan extension but also leads to a modification in inflorescence development and delayed both flowering and onset of senescence under long-day conditions (20h light/4h night) (Baena-Gonzalez *et al.*, 2007). Both OsSnRK1 (Rice) and AtSnRK1 (*Arabidopsis*) show the ability to delay aging by induction of the expression of senescence-related genes such as chlorophyll a/b binding protein 2 (CAB2), senescence 1 (SEN1) and senescence-associated gene12 (SAG12) in WT, contrary to what happened with the transgenic plants expressing inactive forms of SnRK1 (Cho *et al.*, 2012; Tsai and Gazzarrini, 2012)

SnRK1 complex plays an important role in virtually all development stages. SnRK1.1 deficiency results in deficient seed maturation and germination (Zhang *et al.*, 2001; Lu *et al.*, 2007; Rosnoblet *et al.*, 2007), affect embryo development and cotyledon growth (Radchuk *et al.*, 2006; Radchuk *et al.*, 2010) and causes abnormal pollen development and male sterility in barley (*Hordeum vulgare*) (Zhang *et al.*, 2001). SnRK1.1 overexpression seedlings present improved primary root growth and development even under low light with limited energy source (Baena-Gonzalez *et al.*, 2007).

In plants, stresses generally induce alteration in both carbon and nitrogen metabolisms and particularly, frequently compromise photosynthesis and/or respiration resulting in a decrease of the cellular energy levels and preventing plant growth (Baena-Gonzalez and Sheen, 2008; Jossier *et al.*, 2009). For example, specific stress conditions such as flooding (oxygen-limited environments) impairs mitochondrial respiration (Baena-Gonzalez and Sheen, 2008) and plant cells must shift metabolism to fermentation (Rolland *et al.*, 2002).

### *1.2.3.1. SnRK1 activity regulation*

Similarly to what is observed in animals and yeast, activation of SnRK1.1 is dependent on its phosphorylation by upstream kinases (figure 3). GRIK1 and GRIK2 (geminivirus rep-interacting kinases) also called AtSnAK2 and 1 (*Arabidopsis* SnRK1-activating kinases) respectively, have been shown to phosphorylate the SnRK1.1 catalytic subunit on $Thr^{175}$ (Shen *et al.*, 2009; Crozet *et al.*, 2010). They are phylogenetically related to yeast Sak1, Tos3 and Elm1 and mammalian LKB1 and CaMKKβ. GRIKs complements the tos3Δpak1Δelm1Δ triple mutant phenotype (Shen *et al.*, 2009; Crozet *et al.*, 2010). Moreover, mammalian LKB1 can phosphorylate and activated plant SnRK1 indicating that this pathways have been conserved throughout evolution (Crozet *et al.*, 2010). However GRIK-mediated activation of SnRK1 is not affected by 5'-AMP nor by $Ca^{2+}$ levels. Interestingly, GRIK proteins are founded exclusively in young tissues where occur synthesis of DNA and cell division meaning that GRIK-SnRK1 interaction may play a regulatory role in young tissues and geminivirus-infected cells which are two environments where there is high metabolic requirement. Thus this interaction may ensure energy and nutrient supplies for cell growth and plant cell cycle control (Shen *et al.*, 2009).

Protein Phosphatase (PP) activity in plants, as in mammals, is necessary to reduce the activity of Protein kinases by dephosphorylation (Sheen, 1998; Wang *et al.*, 2007; Umezawa *et al.*, 2009; Guo *et al.*, 2010; Danquah *et al.*, 2014). The clade A type 2C phosphatases (PP2Cs) belong to the Protein Ser/Thr Phosphatase group and are implicated in stress response signalling in *Arabidopsis.* PP2Cs block ABA-induced gene expression by dephosphorylation and inactivation of SnRK2s (Sheen, 1998; Umezawa *et al.*, 2009; Guo *et al.*, 2010). More recently, at least two of the clade A PP2Cs, PP2CA and ABI1, were shown to also inhibit SnRK1 activity *in vitro* and *in vivo* by dephosphorylation, acting as the negative regulators of the complex (figure 3) (Rodrigues *et al.*, 2013).

### *1.2.3.2. SnRK1 downstream targets*

SnRK1 plays an important role in the re-establishment of the homeostasis in plants through activation/repression of over 1000 stress-related genes (Hardie, 2007; Polge and Thomas, 2007; Baena-Gonzalez, 2010; Akpinar *et al.*, 2012; Cho *et al.*, 2012) and genes involved in metabolic and transcriptional changes (Baena-Gonzalez *et al.*, 2007; Baena-

Gonzalez and Sheen, 2008; Shen *et al.*, 2009; Confraria *et al.*, 2013). Several types of proteins are implicated in this specific response like phosphatases, histones, histone deacetylases, kinases and calcium modulator, and also several TFs that will act directly in chromatin, contributing to the plant response (figure 3) (Baena-Gonzalez *et al.*, 2007; Baena-Gonzalez and Sheen, 2008; Ghillebert *et al.*, 2011; Cho *et al.*, 2012).

HMG-CoA reductase (3-hydroxy-3-methylglutaryl-CoA reductase also presents in mammals), nitrate reductase (NR), sucrose phosphate synthase (SPS) (Sugden *et al.*, 1999b) Trehalose-6-Phosphate Synthase 5 (TPS5) (Zhang *et al.*, 2009; Nunes *et al.*, 2013b) and nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase (Sugden *et al.*, 1999b; Laurie and Halford, 2001; Li *et al.*, 2009; Shen *et al.*, 2009; Vanesa Piattoni *et al.*, 2011; Coello and Martinez-Barajas, 2014) are some of the few downstream targets of SnRK1 that have been identified. It is interesting to notice that these proteins are involved in carbon and nitrogen metabolisms - the HMG-CoA reductase is involved in isoprenoids biosynthesis; the NR is the key enzyme in nitrogen assimilation which reduces nitrate to nitrite; SPS is involved in sucrose biosynthesis in leaves (Sugden *et al.*, 1999b; Laurie and Halford, 2001); TPS coverts glucose-6-phosphate (G6P) and UDPglucose (UDPG) to trehalose-6-phosphate (T6P) (Harthill *et al.*, 2006); and the nonphosphorylating glyceraldehyde-3-phosphate dehydrogenase catalyses a specific oxidation reaction to generate NADPH (Habenicht, 1997)

The TFs work as downstream regulators of the stress-response and only a few of them were identified as substrates of SnRK1 (figures 3) (Baena-Gonzalez *et al.*, 2007; Cabello *et al.*, 2014). Some of the *Arabidopsis* TFs were identified as suppressors of the yeast *snf4* mutation and they are closely related to the response to cold, salt and drought (Baena-Gonzalez and Sheen, 2008; Baena-Gonzalez, 2010). The TFs regulated by SnRK1 belong to S-group of bZIP TFs: bZIP1, bZIP2/GBF5, bZIP11/ATB2, bZIP44 and bZIP53 (figure 3). Interestingly The S-group bZIP TFs are repressed by presence of sucrose (Baena-Gonzalez, 2010).

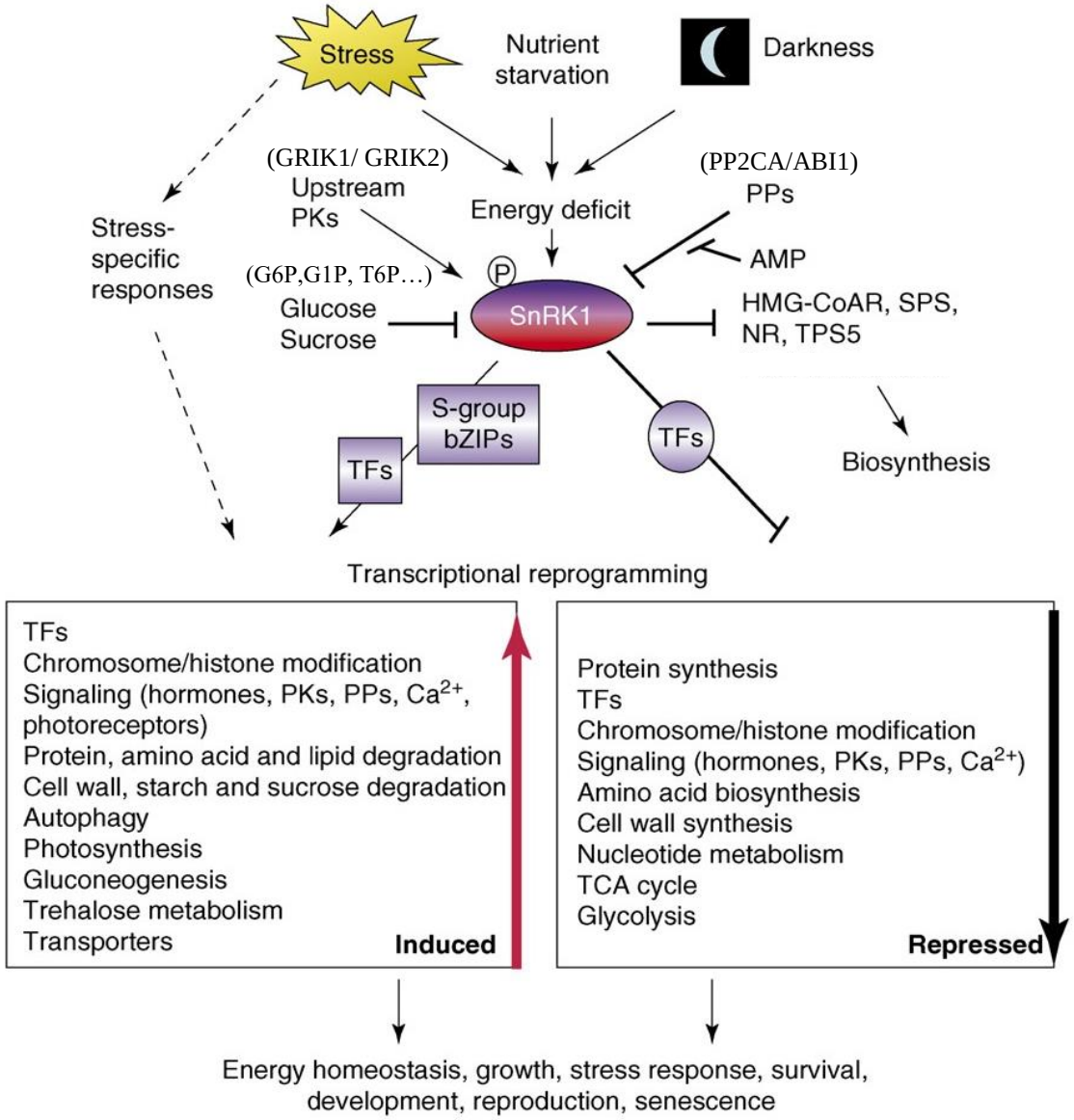


***Figure 3: An overview of SnRK1 Complex activity.***
*Several types of stress (hypoxia, herbicides, drought, salt, flooded soil, darkness, sugar depletion and other stresses) lead to an energy deficit signal in cells, that triggers the activation by phosphorylation of SnRK1.1/1.2 by upstream kinases. Activated SnRK1 initiates a transcriptional reprogramming at several levels which is partly mediated by the S-class of bZIP transcription factors.to repress biosynthetic pathways and promote catabolic processes and photosynthesis, to achieve the re-establishment of the plant energy homeostasis. Besides contributing to the maintenance of cellular energy homeostasis and tolerance to stress, SnRK1 has profound effects at the whole-organism level, influencing growth, survival, reproduction and senescence. PPs and some sugars are able to inhibit SnRK1 activity although by different pathways.*
*Abbreviations: TCA-tricarboxylic acid cycle (source: Baena-Gonzalez and Sheen, 2008)*

## Aim:

The aim of this work was to identify novel protein interactors of the β1 subunit of the SnRK1 complex, using a yeast two hybrid screen, and in this way identify novel substrates of SnRK1, therefore contributing to a better knowledge of the energy stress signalling pathway in plants.

# Materials and Methods

# 2. Materials and Methods

## 2.1. Construction of Plasmids

The construction with the binding domain of GAL4 (BD) fused to SnRK1β1 to be used as bait in the yeast two-hybrid (Y2H) assays was generated in the pGBKT7 vector (Clontech). To this purpose the coding sequence (CDS) of SnRK1β1 was amplified with specific primers (table I) using Q5 High-Fidelity DNA Polymerase (NEB) according to the manufacturer's instructions, in a 50µl reaction volume. The PCR reaction was performed using 35 cycles with an annealing temperature of 55°C. After confirming, by agarose gel electrophoresis, that a DNA fragment with the expected size was obtained, the PCR product (the remaining 45µl) was purified using a phenol-chloroform extraction (Green MR and Sambrook JR., 2012) and digested with the respective restriction enzymes (table I).

The fusions of the activation domain of GAL4 (AD) with specific proteins to be used as preys to test their interaction with SnRK1β1 were generated in pGADT7. To this end the CDS of each protein was amplified by PCR with specific primers (table I) and cloned into pGADT7 as described in the previous paragraph.

**Table I:** ***Sequence of the primers used in this work for PCR amplifications.***
*The restriction sites are represented in lowercase letters.*

| AGI | Name of primer | Primer sequence (5'- 3') | Vector | Restriction sites |
|---|---|---|---|---|
| **AT5G21170** | SnRK1β1Fw<br>SnRK1β1Rev | tttgaattcATGGGAAATGCGAACGGCAA<br>tttgtcgacTTACCGTGTGAGCGGTTTGTAG | pGBKT7 | **EcoRI/ SalI** |
| **AT5G25220** | KNAT3 Fw<br>KNAT3 Rev | tttgaattcATGGCGTTTCATCACAATCATC<br>aaactcgagCTACGCGAACCGCTCTCTTC | pGADT7 | **EcoRI/ XhoI** |
| **AT5G11060** | KNAT4 Fw<br>KNAT4 Rev | tttgaattcATGGCGTTTCATAACAATCAC<br>aaactcgagTCAACGGTCTCTTCCGCTGT | pGADT7 | **EcoRI/ XhoI** |
| **AT4G32040** | KNAT5 Fw<br>KNAT5 Rev | tttgaattcATGTCGTTTAACAGCTCCCACC<br>aaactcgagCTACGACTTCCCGGTCCGTT | pGADT7 | **EcoRI/ XhoI** |
| **AT1G62990** | KNAT7 Fw<br>KNAT7 Rev | tttgaattcATGCAAGAAGCGGCACTAGG<br>aaactcgagTTAGTGTTTGCGCTTGGACTTC | pGADT7 | **EcoRI/ XhoI** |
| **AT2G47580** | U1A Fw<br>U1A Rev | tttgaattcATGGAGATGCAAGAGGCTAATC<br>aaactcgagCTATTTCTTGGCATACGTGATG | pGADT7 | **EcoRI/ XhoI** |
| **AT2G30260** | U2B" Fw<br>U2B" Rev | tttgaattcATGTTAACGGCAGATATACCAC<br>aaactcgagTCATTTCTTGGCGAAAGAGATG | pGADT7 | **EcoRI/ XhoI** |
| **AT1G06960** | AT1G06960 Fw<br>AT1G06960 Rev | tttgaattcATGTTAACGGCCTGATATACCAC<br>aaactcgagTCACTTCTTGGCAAAAGAGACG | pGADT7 | **EcoRI/ XhoI** |
| **AT5G04140** | GLU1 Fw<br>GLU1 Rev | tttgaattcATGGCGATGCAATCTCTTTCC<br>aaagtcgacCTAAGCCGATTGAAATGTGACT | pGADT7 | **EcoRI/ SalI** (insert)<br>**EcoRI/XhoI** (vector) |
| **AT2G41220** | GLU2 Fw<br>GLU2 Rev | tttgaattcATGGCTCTACAGTCTCCCGG<br>aaagtcgacTTACTTCTCTGCTAATGTGCTTG | pGADT7 | **EcoRI/ SalI** (insert)<br>**EcoRI/XhoI** (vector) |

## 2.2. Yeast two-hybrid screen

### 2.2.1. Transformation of Y2HGold with pGBKT7-SnRK1β1

To identify proteins encoded by the *Arabidopsis* genome that interact with SnRK1β1, an Y2H screen was performed using a Mate & Plate™ Library - Universal *Arabidopsis* (Clontech), and a normalized library into pGADT7-RecAB (Clontech) constructed from mRNA isolated from 11 Arabidopsis tissues, mixed in equal quantities and transformed into yeast strain Y187. The bait construction BD-SnRK1β1 was used to transform Y2HGold yeast cells (Clontech), using the Yeastmaker Yeast Transformation System 2 (Clontech) according to the manufacturer's instructions. Briefly, 100ng of pGBKT7- SnRK1β1 were combined with 50µg of Yeastmaker Carrier DNA (Clontech), 50µl of yeast competent cells and 500µl of PEG/LiAc (40% PEG 3350, 0.1M lithium acetate in TE buffer) in a sterile tube (1.5 ml). After were added and mixed to join incubation at 30°C for 30 min, 20µl of DMSO were added and the mix was incubated at 42° for 20 min. After that, the tube was centrifuged (2000rpm, 5min) to obtain the pellet and the supernatant was removed. The pellet yeast cells

were resuspended in 100µl and plated onto a 100mm plate with SD -Trp (SD-W) agar medium.

### 2.2.2. Mating and selection of clones

The screening of the Y2H library was performed using the Matchmaker® Gold Yeast Two-Hybrid System (Clontech) according to the manufacturer's instructions. Briefly, 45ml of 2X YPDA (supplemented with 50µg/ml kanamycin) were inoculated with 5ml of a bait culture (with a cell density higher than $10^8$ cells/ml) and an aliquot (1 ml) of the Y2H library, and incubated for 24h at 30°C with slowly shaking (40 rpm). After incubation, the culture was centrifuged (1000g for 10min). The supernatant was removed and the cell pellet was resuspended in 10ml of water. The cells were spread onto 150 mm diameter plates (200µl per plate) containing SD medium lacking leucine and tryptophan (SD-L-W) supplemented with X-α-GAL (10 mg/L) and Aureobasidin A (AbA) (200 µg/L) and the plates were incubated for 5 days at 30ºC. To control the mating efficiency and the number of diploids formed 100 µl of successive dilutions $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$ of the resuspended cells were spread onto SD-L, SD-W and SD-L-W (SD-2) media plates and incubated for 3 days at 30ºC (Table II).

The blue colonies that grew on SD-L-W supplemented with X-α-GAL and AbA were patched out onto higher stringency SD medium lacking leucine, tryptophan, adenine and histidine (SD-L-W-A-H), also supplemented with X-α-GAL and AbA. Those colonies that continued to report blue colour were used for further analysis and identification of proteins that possibly interact with SnRK1β1.

***Table II: Measurement of the viability of the Prey Library, the Bait and diploids expressing interacting proteins.***
*After proceed the mating between the two strains used, 100µl were plated in different media with four dilutions ($10^{-1}$; $10^{-2}$; $10^{-3}$; $10^{-4}$).*

| ***Strain*** | **Plate on SD Minimal Agar Medium** | **Selects for** |
|---|---|---|
| *Y2HGold [pGBKT7-53 + SnRK1β1]* ***x*** *Y187[pGADT7-T + library] with normalized library* | SD -L | pGADT7-T |
| | SD -W | pGBKT7-53 |
| | SD -L-W | Diploids containing pGBKT7-53 and pGADT7-T |
| | SD -L/-W/ X-α-Gal/AbA | Diploids that have also activated AbA resistance and α-galactosidase through protein-protein interactions |

## 2.2.3. Determination of transformation and mating efficiency

The total volume obtained after 20hr of mating (pelleted cells plus 10ml of medium) was measured to determine the mating efficiency and the number of colonies diploids. The number of screened clones (diploids) was calculated considering the total volume used, the plating volume, the number of cfu/ml of diploids and the dilution considered (Appendix 1). The mating efficiency was calculate using the ratio between number of cfu/ml of diploids and No. of cfu/ml grew in SD-Leu (SD-L) agar plate.

## 2.2.4. Yeast plasmid DNA extraction

The blue colonies that grown in SD -L-W-A-H (SD-4) medium supplemented with X-α-Gal and AbA, were used to inoculate 2ml of SD -L-W liquid medium that was incubated for 48h at 30ºC with shaking. DNA extraction was performed using a modified smash and grab method (Hoffman and Winston, 1987). After incubation cells were pelleted by centrifugation (1 min, 10000g) and resuspended in 200µl of lysis buffer (10mM Tris, pH 7.5; 1mM EDTA; 100mM NaCl; 1% of SDS; 2% of 100XTriton). After adding 200µl of glass beads and 200µl of phenol-chloroform (50:50) the mix was vortexed for 4 min. Then, after 200µl of TE was added and the mix was vortexed again for 1 minute. After centrifugation at RT for 5 min (13000g) the aqueous phase was transferred to another centrifuge tube and the DNA was precipitated adding 1/10 volume of NaAc 3M, pH=5.2 plus 3 volumes of 100% ethanol and incubating at -20°C for 30 min. Next, the mix was centrifuged at 4°C for 20 min

(13000g) and the supernatant was discarded. The pellet was washed with 750µl of 70% Ethanol and dried. Finally the DNA was resuspended in 10µl of sterile water.

The cDNA cloned in each of the rescued plasmids was amplified by PCR using the primers AAATATTCGATGATGAAGATACCCCACCAAACCCA and TTTAGTGAACTTGCGGGGTTTTTCAGTATCTACGAT, using the OneTaq® DNA Polymerase according to the manufacturer's instructions in a 50µl of reaction volume. The PCR reaction was performed using 35 cycles with denaturation at 94ºC for 30 sec an annealing/extension at 68°C for 4 min. The amplification product was analyzed by agarose gel electrophoresis. Sequencing of the PCR products was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher) according to the manufacturer's instructions.

## 2.3. Binary interactions

To study the possible interaction between SnRK1β1 and specific proteins, the Y2H technique was used. The cotransformation of yeast was done mainly according to the lithium acetate protocol (Gietz *et al.*, 1997). Briefly, 25µl of competent cells Y2HGold were mixed with 1µg of each plasmid construction (bait and prey) and 700µl of PEG/LiAc. The mix was incubated at 30°C for 30 min and after further incubated 20min at 42ºC. Next, the cells were pelleted by centrifugation for 5 min (2000g) and the supernatant was discarded. The cells pellet was resuspended in 100µl of sterile water and spread into SD -L-W agar plate that were incubated for 2 days at 30°C. Next, 3 colonies were randomly picked and used to inoculate 2ml of SD -L-W liquid medium that was incubated at 30ºC until the culture was saturated (48h). Finally, the interaction was determined by growth assay on SD -L-W-A-H agar medium, 3µl of $10^{-1}$, $10^{-2}$ and $10^{-3}$ dilutions of the saturated cultures were spotted onto the plates. As control the growth on SD -L-W medium was also tested.

## 2.4. Auxiliaries software's

### 2.4.1. The Arabidopsis Information Resource (TAIR)

Identification of the sequences obtained was performed using the online BLAST program of the Arabidopsis Information Resource (TAIR) (https://www.arabidopsis.org/Blast/index.jsp).

### 2.4.2. The MIPS Functional Catalogue Database (FunCatDB)

Functional characterization of the identified genes was done using the online MIPS Functional Catalogue Database (FunCatDB) (http://mips.helmholtz-muenchen.de/funcatDB/). Additionally this program provides statistics of the functional distribution of a given set of genes (Ruepp *et al.*, 2004). In this case a set of 64 proteins was submitted and compare with the whole genome of *Arabidopsis thaliana* (Taxonomy ID: 3702)

### 2.4.3. The Arabidopsis Protein Phosphorylation Site Database (PhosPhAt 4.0)

The information about the experimentally-identified phosphorylation sites of proteins were obtained using the PhosPhAt 4.0 database (http://phosphat.uni-hohenheim.de/phosphat.html). (Heazlewood *et al.*, 2008; Durek *et al.*, 2010; Zulawski *et al.*, 2013)

# Results

# 3. Results

## 3.1. Identification of SnRK1β1 protein interactors

As previously mentioned in the introduction, the protein-protein interaction is one of the most common processes involved in the response to a certain stimuli (Baena-Gonzalez *et al.*, 2007; Baena-Gonzalez and Sheen, 2008). Besides the importance of α-subunits in the AMPK/SNF1/SnRK1 complex, the regulatory subunits (β- and γ-subunits) are also very important to activate the complex, to select the downstream targets, and to establish relevant interactions that are important for the regulation of gene expression during stress (Lovas *et al.*, 2003; Gissot *et al.*, 2006; Pierre *et al.*, 2007; Li *et al.*, 2009; Baena-Gonzalez, 2010; Ramon *et al.*, 2013). Despite that, the knowledge about the molecular components involved in the stress signalling pathway is scarce. In order to identify new proteins that possibly interact with SnRK1β1 and, in this way, are involved in the stress-response, an Y2H screen was performed using SnRK1β1 as bait.

The mating efficiency was 4.4%, within the expected limits according to the manufacturer of the kit used to perform the screen (2% to 5%). The number of diploid clones screened was equal to 4 million, four times the minimal number of clones recommended by the manufacturer of the kit used (see Appendix 1).

The blue colonies that grew in (SD -L-W) agar plates (150 mm plates) supplemented with X-α-Gal and AbA were patched out onto a higher stringency medium also supplemented with X-α-Gal and AbA (SD -L-W-A-H agar plates). Of the 297 yeast colonies that continued to grow and presented blue color in this new medium, 72 were randomly selected to be further analyzed. The remaining colonies were used to inoculate SD-L-W liquid medium. After incubation for 2 days at 30ºC, glycerol was added (25% final concentration) and they were stored at -80 °C.

The analysis of the 72 selected colonies allowed the identification of 64 different proteins that possibly interact with SnRK1β1 (Table III). Two of these proteins, SnRK1.2 (KIN11) (Baena-Gonzalez *et al.*, 2007; Fragoso *et al.*, 2009; Williams *et al.*, 2014) and Hsp70 (Slocombe *et al.*, 2004) have previously been identified as SnRK1β1 interactors, suggesting that this screen was indeed able to identify SnRK1β1 interacting proteins with SnRK1β1.

***Table III: Putative SnRK1 β1-subunit interactors identified in this Y2H screen.***

| N | AGI code | Description | N | AGI code | Description |
|---|---|---|---|---|---|
| 1 | At5g39380 | Plant calmodulin-binding protein-related | 33 | At1g75460 | ATP-dependent protease La domain protein |
| 2 | At3g10910 | DAFL1 | 34 | At1g51200 | A20/AN1-like zinc finger family protein |
| 3 | At1g19800 | TGD1 | 35 | At3g22320 | RPB5A |
| 4 | At2g40410 | CAN2 | 36 | At2g33793 | Unknown protein |
| 5 | At5g08300 | Succinyl-CoA ligase, alpha subunit | 37 | At1g23460 | Pectin lyase-like superfamily protein |
| 6 | At3g11530 | VPS55 family protein | 38 | At5g42990 | UBC18 |
| 7 | At1g77120 | ATADH | 39 | At5g59120 | Subtilase 4.13 |
| 8 | At3g11830 | TCP-1/Cpn60 chaperonin family protein | 40 | At2g43130 | ARA4 |
| 9 | At5g04140 | GLU1 | 41 | At5g15090 | VDAC3 |
| 10 | At5g06130 | Chaperone protein dnaJ-related | 42 | At1g47380 | Protein phosphatase 2C family protein |
| 11 | At3g29160 | SnRK1.2 | 43 | At3g19710 | BCAT4 |
| 12 | At1g69080 | Adenine nucleotide alpha hydrolases-like superfamily protein | 44 | At1g61600 | DUF1262 |
| 13 | At5g08280 | RUGOSA 1 | 45 | At1g05060 | Unknown protein |
| 14 | At1g04410 | C-NAD-MDH1 | 46 | At2g44750 | TPK2 |
| 15 | At5g17770 | CBR1 | 47 | At5g02490 | Hsp70-2 |
| 16 | At4g26530 | ATFBA5 | 48 | At1g70600 | Ribosomal protein L18e/L15 superfamily protein |
| 17 | At4g17730 | SYP23 | 49 | At5g65160 | TPR14 |
| 18 | At2g47580 | Spliceosomal protein U1A | 50 | At4g16330 | 2OG and Fe(II)-dependent oxygenase superfamily protein |
| 19 | At5g64400 | protein_coding | 51 | At5g67260 | CYCD3;2 |
| 20 | At1g78060 | Glycosyl hydrolase family protein | 52 | At1g31817 | NFD3 |
| 21 | At5g16800 | NAT superfamily protein | 53 | At1g53590 | Calcium-dependent lipid-binding |
| 22 | At1g53040 | DUF616 | 54 | At3g07820 | Pectin lyase-like superfamily protein |
| 23 | At1g13635 | DNA glycosylase superfamily protein | 55 | At3g47670 | Plant invertase/Pectin methylesterase inhibitor superfamily protein |
| 24 | At3g54540 | ATGCN4 | 56 | At5g06340 | Nudix hydrolase homolog 27 |
| 25 | At5g54390 | ATAHL | 57 | At3g10920 | MSD1 |
| 26 | At4g32040 | KNAT5 | 58 | At4g12340 | copper ion binding |
| 27 | At5g03690 | ATFBA4 | 59 | At1g11125 | unknown protein |
| 28 | At5g06043 | unknown protein | 60 | At4g34980 | SLP2 |
| 29 | At4g01840 | KCO5 | 61 | At1g30380 | Photosystem I subunit K |
| 30 | At4g05060 | PapD-like superfamily protein | 62 | At3g32930 | unknown protein |
| 31 | At3g12130 | zinc finger (CCCH type) family protein | 63 | At4g16360 | SnRK1β2 |
| 32 | At5g48880 | PKT1 | 64 | At1g73655 | FKBP-like peptidyl-prolyl cis-trans isomerase family protein |

### 3.1.1. Functional categorization

A functional categorization of the 64 proteins identified was performed using the MIPS FunCat (Ruepp *et al.*, 2004). A large number of these proteins are involved in metabolism (33 proteins), namely of sugars, phosphates and fatty acids, in energy processes (14 proteins) like glycolysis, gluconeogenesis and oxidation of fatty acids, and in nutrient starvation response (4 proteins) (table IV and Table III in Appendix 2). Some other of the identified proteins are related to binding functions (27 proteins), cellular transport (11 proteins) and cell defense (13 proteins) (table IV and Table III in Appendix 2), being that all these proteins may be included in different categories. Taken together, these results are clearly in line with the importance of SnRK1 complex in the response to energy stress and the re-establishment of energy homeostasis.

***Table IV: The main overrepresented FunCat categories with a P-value < 0.05.***
*The Categories in bold are the main category, the others are sub-categories.*

| FUNCTIONAL CATEGORY | Rel. SET (%) | Rel. GENOME (%) | P-VALUE |
|---|---|---|---|
| **01 METABOLISM** | 39,2 | 21,2 | **1,24E-04** |
| 01.04 phosphate metabolism | 11,9 | 4,2 | **2,76E-03** |
| 01.05 C-compound and carbohydrate metabolism | 20,2 | 8,21 | **4,20E-04** |
| 01.05.02 sugar, glucoside, polyol and carboxylate metabolism | 10,7 | 1,91 | **3,38E-05** |
| 01.05.02.07 sugar, glucoside, polyol and carboxylate catabolism | 8,33 | 1,04 | **2,96E-05** |
| 01.05.25 regulation of C-compound and carbohydrate metabolism | 3,57 | 0,65 | **1,80E-02** |
| 01.06.05 fatty acid metabolism | 7,14 | 0,61 | **1,39E-05** |
| 01.07 metabolism of vitamins, cofactors, and prosthetic groups | 4,76 | 1,6 | **4,64E-02** |
| 01.07.01 biosynthesis of vitamins, cofactors, and prosthetic groups | 3,57 | 0,95 | **4,63E-02** |
| **02 ENERGY** | 16,6 | 4,44 | **1,89E-05** |
| 02.01 glycolysis and gluconeogenesis | 4,76 | 0,88 | **6,62E-03** |
| 02.07 pentose-phosphate pathway | 4,76 | 0,29 | **1,12E-04** |
| 02.10 tricarboxylic-acid pathway (citrate cycle, Krebs cycle, TCA cycle) | 2,38 | 0,34 | **3,47E-02** |
| 02.25 oxidation of fatty acids | 7,14 | 0,21 | **3,14E-08** |
| 14.07.03 modification by phosphorylation, dephosphorylation, autophosphorylation | 5,95 | 2,04 | **2,90E-02** |
| **16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT** | 32,1 | 22,2 | **2,35E-02** |
| 18.01.01 regulation by modification | 5,95 | 1,29 | **4,82E-03** |
| 18.02.09 regulator of transcription factor | 3,57 | 0,93 | **4,42E-02** |
| 20.03.01.01 ion channels | 2,38 | 0,38 | **4,26E-02** |
| 20.09.07.27 vesicle fusion | 2,38 | 0,28 | **2,35E-02** |
| 20.09.16 cellular export and secretion | 3,57 | 0,95 | **4,63E-02** |
| 20.09.16.09 vesicular cellular export | 2,38 | 0,25 | **1,91E-02** |
| 20.09.18.09 vesicular cellular import | 3,57 | 0,73 | **2,38E-02** |
| 20.09.18.09.01 endocytosis | 3,57 | 0,73 | **2,38E-02** |
| 30.01.05 enzyme mediated signal transduction | 7,14 | 2,45 | **1,74E-02** |
| 30.01.09 second messenger mediated signal transduction | 4,76 | 0,91 | **7,42E-03** |
| 30.01.09.07 cAMP/cGMP mediated signal transduction | 3,57 | 0,04 | **5,47E-06** |
| 32.01 stress response | 15,4 | 6,31 | **2,27E-03** |
| 32.01.11 nutrient starvation response | 4,76 | 0,12 | **3,87E-06** |
| 32.07.03 detoxification by modification | 2,38 | 0,34 | **3,47E-02** |
| 32.07.07.07 superoxide metabolism | 2,38 | 0,08 | **2,60E-03** |
| 34.07 cell adhesion | 3,57 | 0,21 | **8,07E-04** |
| 34.07.02 cell-matrix adhesion | 3,57 | 0,02 | **8,51E-07** |
| 40.01.05 growth regulators / regulation of cell size | 3,57 | 0,55 | **1,15E-02** |
| 40.20 cell aging | 5,95 | 0,35 | **1,20E-05** |
| **43 CELL TYPE DIFFERENTIATION** | 7,14 | 1,74 | **3,58E-03** |
| 43.01 fungal/microorganismic cell type differentiation | 7,14 | 1,74 | **3,58E-03** |
| 43.01.03 fungal and other eukaryotic cell type differentiation | 7,14 | 1,74 | **3,58E-03** |
| 43.01.03.05 budding, cell polarity and filament formation | 5,95 | 0,86 | **8,15E-04** |

## 3.2. Yeast-two Hybrid System

Considering their relevance in stress response and/or their relationship with the energy stress response, three different proteins were chosen to further analysis: the Spliceosomal protein U1A (At2g47580); KNOTTED1-like homeobox gene 5, KNAT5 (At4g32040); and Glutamate Synthase 1, GLU1 (At5g04140). The U1A and KNAT5 proteins are involved in transcription and DNA processing (Forment *et al.*, 2002; Scofield *et al.*, 2008), while GLU1 is related to metabolism having a crucial role in nitrogen, glutamate and amino acids metabolisms and is also related to stress response (Kissen *et al.*, 2010; Hirel *et al.*, 2011; Aliyev, 2012; Kangasjarvi *et al.*, 2012). The reconfirmation of the interaction of those protein with SnRK1β1 was performed using the Y2H system. Additionally, the interaction of

other proteins of the same families with SnRK1β1, was also tested using the same method: U2 Small Nuclear Ribonucleoprotein B, U2B" (AT2G30260) and a RNA binding protein (AT1G06960); KNAT3 (AT5G25220), KNAT4 (AT5G11060) and KNAT7 (AT1G62990); and GLU2 (AT2G41220) respectively. All these proteins coding sequence were expressed as a fusion with the GAL4 activation domain (AD) using the pGADT7 vector while the coding sequence of SnRK1β1 was expressed in a fusion with GAL4 DNA-binding domain (BD) using the pGBKT7 vector.

As illustrated in figure 4, the Y2H assay shown that U1A interacts with SnRK1β1 corroborating the observations in Y2H Screen. The other two proteins tested, which are similar to U1A, also interact with the SnRK1β1 in the Y2H system, suggesting that they might be a target of SnRK1 (figure 4).

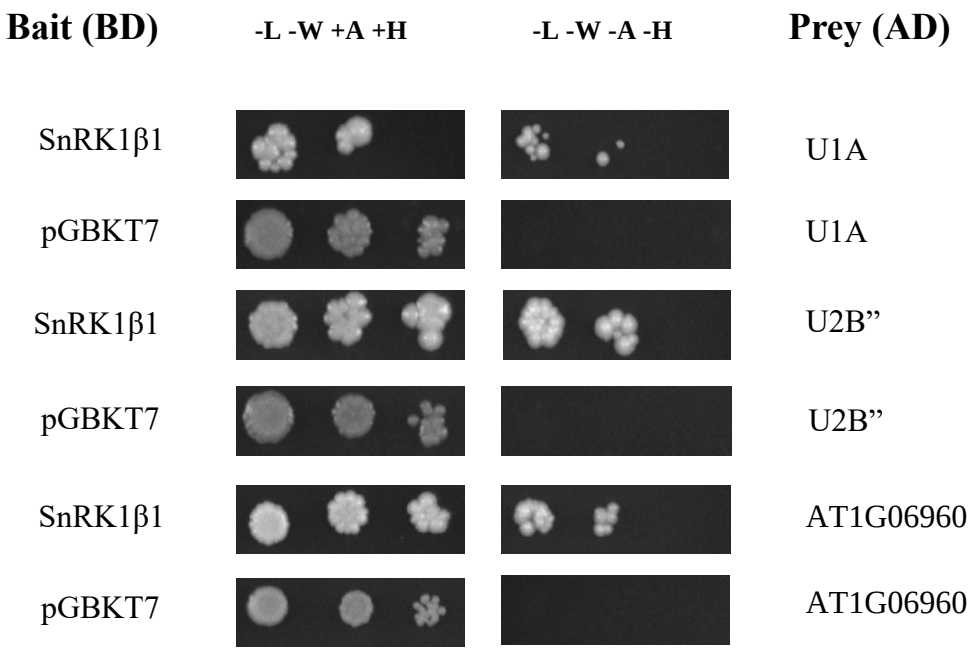


***Figure 4: Protein-protein interaction between SnRK1β1 and U1A, U2B" and AT1G06960 (protein with RNA binding motif).***

*Interaction was determined by growth assay on SD -4 (-L -W -A –H) agar medium. 3µl of three serial dilutions (1/10, 1/100, 1/1000) of each saturated culture were spotted onto the plates. Cells transformed with the empty vector (pGBKT7) were used as a control.*

All KNAT proteins tested were able to interact with β-subunit in the Y2H system (Figure 5). Interestingly, the interaction seems to be stronger in the case of KNAT3 and KNAT7 compare to the interaction with KNAT5 (the protein identified in the screen) and KNAT4 evidencing the weaker interaction with SnRK1β1.

Finally, GLU1 and GLU2 also interact with SnRK1β1 in the Y2H system and GLU2 appears to have a stronger interaction with the bait in comparison with the GLU1 (figure 6).

All controls using the empty pGBKT7 and each of the prey construction were negative, confirming that none of the proteins was able by itself to activate de Y2H reporter genes.

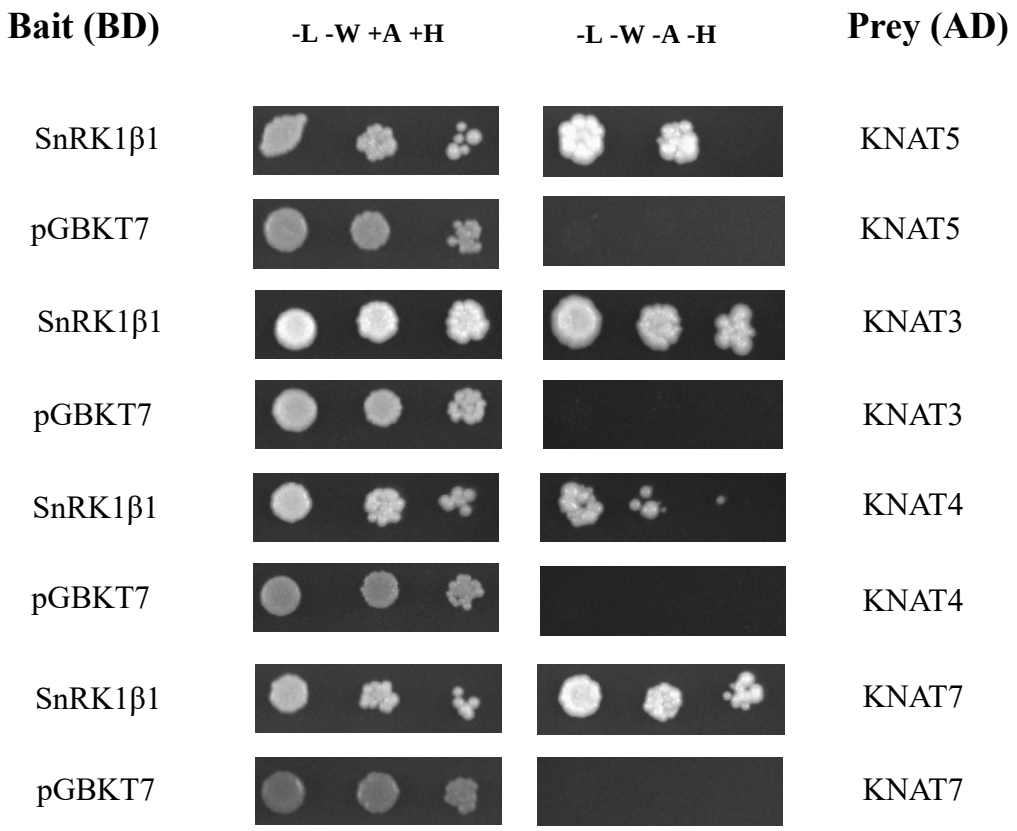


***Figure 5: Protein-protein interaction between SnRK1β1 and KNATs.***
*Interaction was determined by growth assay on SD -4 (-L -W -A -H) agar medium. 3µl of three serial dilutions (1/10, 1/100, 1/1000) of each saturated culture were spotted onto the plates. Cells transformed with the empty vector (pGBKT7) were used as a control*

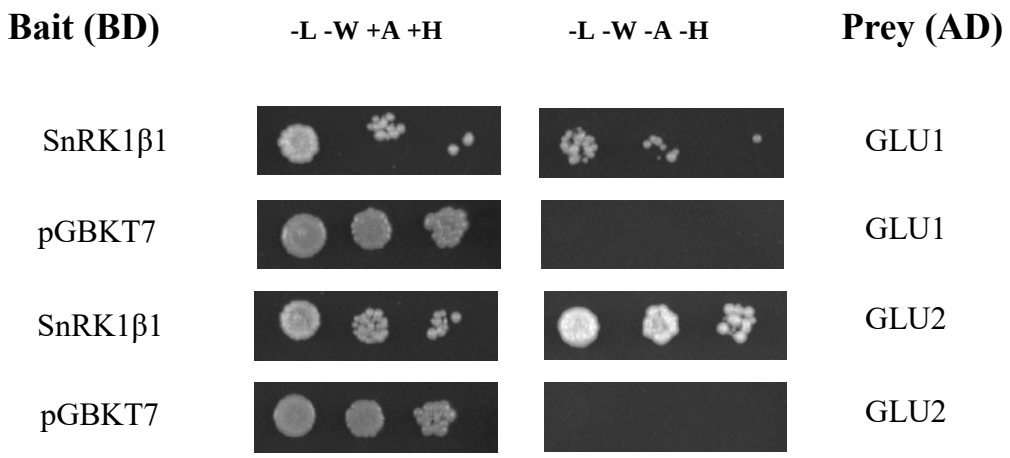


***Figure 6: Protein-protein interaction between SnRK1β1 and GLUs.***
*Interaction was determined by growth assay on SD -4 (-L -W -A -H) agar medium. 3µl of three serial dilutions (1/10, 1/100, 1/1000) of each saturated culture were spotted onto the plates. Cells transformed with the empty vector (pGBKT7) were used as a control.*

Since the β subunits probably facilitate the association of the complex with downstream targets, after the interactions between the proteins previously mentioned and SnRK1β1 were verified, the Y2H system was also used to test the interaction between these proteins and de catalytic subunit. As shown in figure 7, none of the proteins mentioned previously seem to interact with SnRK1.1, suggesting that the interaction of SnRK1β1 with these targets is independent of the presence of SnRK1.1. However, after a longer incubation (7 days at room temperature), some of these proteins, particularly the KNATs, showed evidences of a weak interaction with the α-subunit (SnRK1.1) (figure 8).

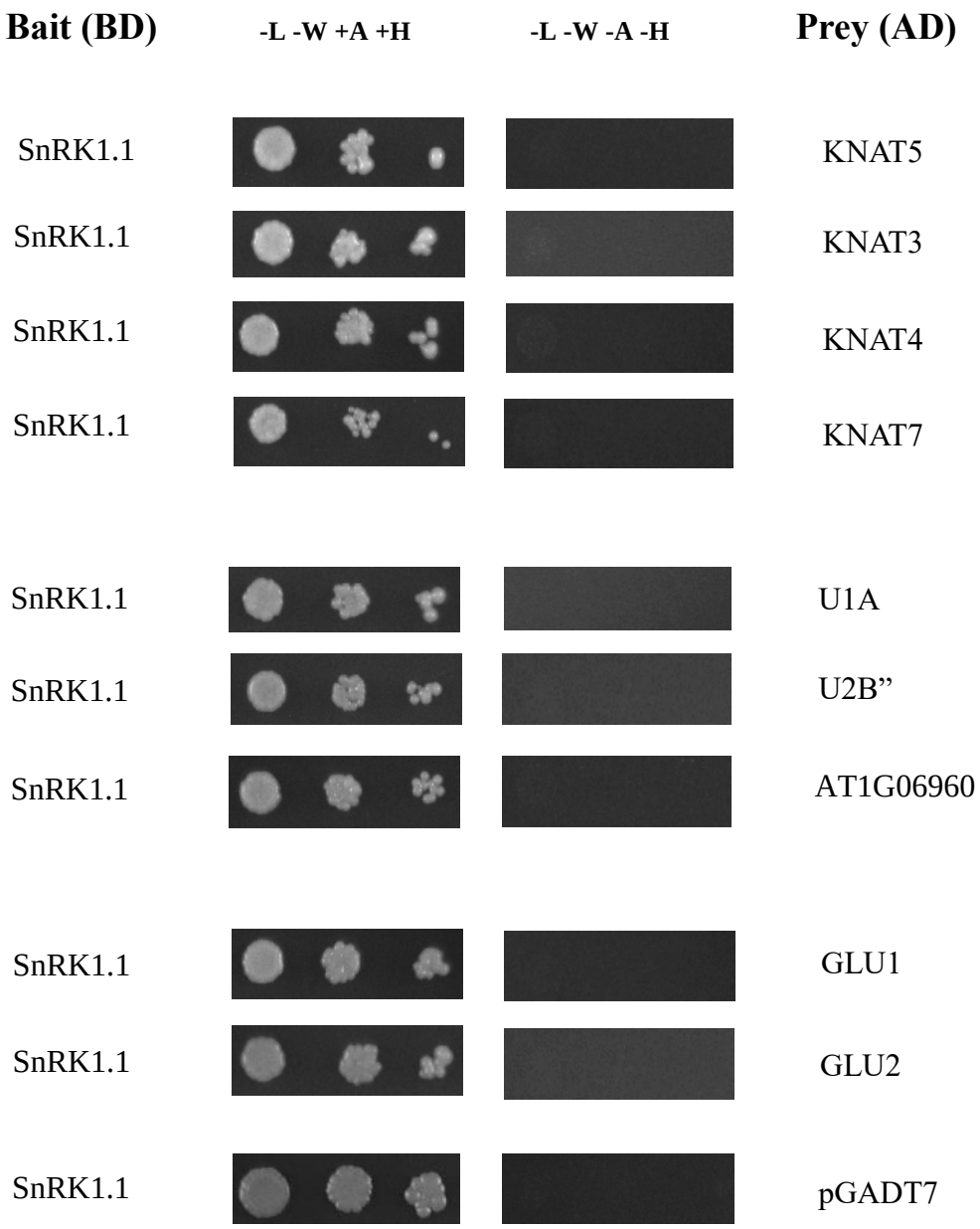


***Figure 7: Protein-protein interaction between SnRK1.1 as a bait with all proteins studied.***

*Interaction was determined by growth assay on SD -4 (-L -W -A –H) agar medium. 3µl of three serial dilutions (1/10, 1/100, 1/1000) of each saturated culture were spotted onto the plates. Cells transformed with the empty vector (pGADT7) were used as a control.*

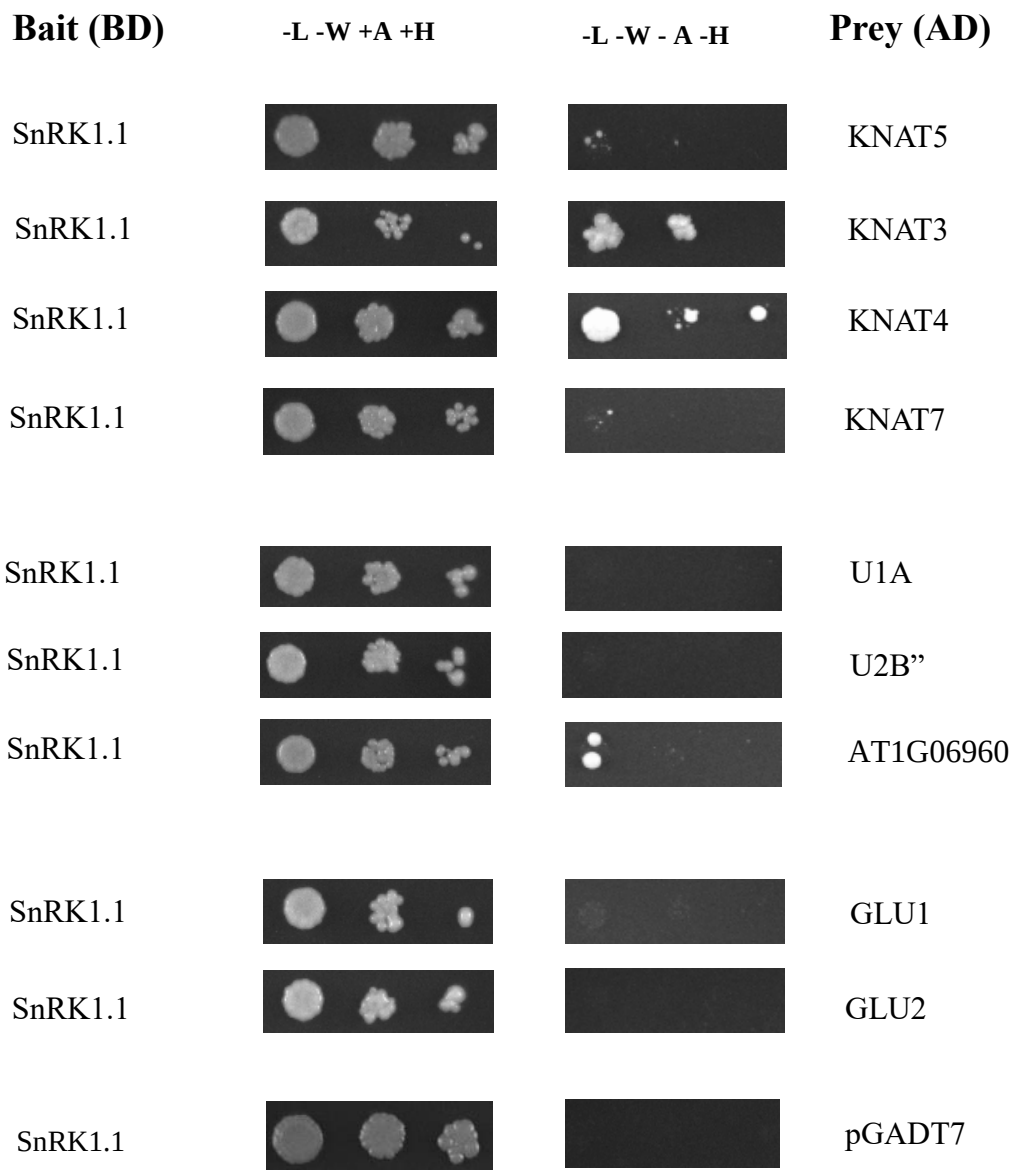


***Figure 8: Protein-protein interaction between SnRK1.1 with all proteins studied under a longer incubation period.***

*Interaction was determined by growth assay on SD -4 (-L -W -A –H) agar medium. 3µl of three serial dilutions (1/10, 1/100, 1/1000) of each saturated culture were spotted onto the plates. The cells growth was verified after 7 days at room temperature.*

# Discussion and Conclusions

# 4. Discussion and conclusions

## 4.1. Identification of SnRK1β1 protein interactors

The way plants respond to biotic and abiotic stresses has been studied for several years (Halford and Hardie, 1998; Serrano *et al.*, 1999; Xiong *et al.*, 2002; Schwachtje *et al.*, 2006; Baena-Gonzalez *et al.*, 2007; Polge and Thomas, 2007; Tuteja, 2007). This study is critical to improve crop production and to minimize the adverse effects of stress on plant growth and development (Serrano *et al.*, 1999; Tuteja, 2007).

The SnRK1 complex has been defined as a central integrator of the stress response. Under stress conditions, SnRK1 activation induces a reprogramming of the transcriptome, by switching on/off specific genes, with a direct impact in the metabolic pathways, energy cycles and carbon sources, that ultimately result in the re-establishment of energy homeostasis and allows the plants to tolerate these adverse conditions (Baena-Gonzalez *et al.*, 2007). Several SnRK1 related genes involved in stress response have already been identified. However, the mechanisms involved are largely unknown and probably most of molecular components remain to be identified (Baena-Gonzalez and Sheen, 2008; Akpinar *et al.*, 2012; Cho *et al.*, 2012; Nietzsche *et al.*, 2014).

In this study, as a primary approach, an Y2H screen was performed using SnRK1β1 subunit. It has been suggested that the beta subunits are involved in the recognition of the SnRK1 substrates (Hedbacker and Carlson, 2008; Li *et al.*, 2009) and so the identification of downstream targets of SnRK1 could be expected. Of the 297 yeast colonies that grew in selective media, 72 were analyzed leading to the identification of 64 proteins that putatively interact with SnRK1. Noteworthy, functional characterization of these proteins shown that more than half of them are related to metabolism, namely of sugars, phosphates and fatty acids. These proteins are also involved in energy processes like glycolysis and gluconeogenesis, Krebs cycle and oxidation of fatty acids (table IV and table III in Appendix 2). These results are consistent with the important role played by SnRK1 in controlling metabolism under stress (Lovas *et al.*, 2003; McKibbin *et al.*, 2006; Pierre *et al.*, 2007; Polge and Thomas, 2007; Baena-Gonzalez and Sheen, 2008; Li *et al.*, 2009; Cho *et al.*, 2012; Crozet *et al.*, 2014) and suggests that some of these proteins are probably downstream targets of SnRK1. These results also support, at least partially,

the proposed facilitating role of the β-subunit in the association with the substrates of the SnRK1 complex.

Of the 64 proteins identified as putative interactors of SnRK1β1, one of them, the Spliceosomal Protein U1A (At2g47580) was selected for further confirmation. U1A present a high sequence similarity with U2B" (AT2G30260) and with a protein containing RNA binding motifs (AT1G06960) so these proteins were also tested to verify if they are able to interact with SnRK1β1. Interestingly, the interaction of SnRK1β1 with U1A seems to be weaker than with U2B" and AT1G06960, although all three interactions evidenced a slowly growth comparing with the control (figure 4). U1A and U2B" are part of the spliceosome which is constituted by four components known as small nuclear ribonucleoprotein particles (snRNPs) (Simpson *et al.*, 1995; Forment *et al.*, 2002). Many additional proteins intervene in the spliceosome acting in the removal of introns from the pre-mRNA with the concomitant ligation of the protein coding sequences (exons), performing the pre-mRNA splicing. This is a fundamental step in the mechanism of regulated gene expression (Forment *et al.*, 2002). In plants, alternative splicing is often associated with abiotic stress since genes with regulatory functions and associated with various stresses are particularly prone to alternative splicing (reviewed in Duque, 2011). The splicing may also influence the efficiency of mRNA nuclear export which is crucial in the cell stress response (Brodsky and Silver, 2000). Additionally, protein:RNA binding, U1A:SLII (Stem-loop II) and U2B":SLIV (Stem-loop IV), are dependent of salt and temperature (Law *et al.*, 2006; Williams and Hall, 2011). More interestingly, the U1A protein was discovered in a screen to isolate plant genes that confer salt tolerance to the yeast *Saccharomyces cerevisiae*, more specifically tolerance to LiCl (Lithium chloride) despite the tolerance be quite weak according to the evaluation of the yeast growth (Forment *et al.*, 2002). The interaction between SnRK1β1 and U1A or U2B", know components of the spliceosome, might represent a novel molecular link between the stress response and the splicing machinery.

Other SnRK1β1 putative interactor that draw our attention was a protein belonging to the KNOTTED-like from Arabidopsis thaliana (KNAT) family. The Y2H assay performed shown that all the tested proteins of this family interact with the β1-subunit (figure 5). KNAT5 was the protein identified in screen but the KNAT3, KNAT4 and KNAT7 have highly sequence similarities with it (Serikawa *et al.*, 1996) being KNAT7 the more divergent one. These proteins present in their structure a homeodomain that acts

as transcription factor and therefore they have a crucial role in development among the eukaryotes (Gehring, 1987; Sakamoto *et al.*, 2001; Kim *et al.*, 2003; Truernit *et al.*, 2006; Truernit and Haseloff, 2007; Scofield *et al.*, 2008; Rutjens *et al.*, 2009). Additionally, homeodomain proteins are capable of turning on or off the expression of cascades of genes, but the DNA binding capacity of these proteins is generally weak. Thus, protein-protein interactions has been shown to be essential to confer high affinity binding of KNAT proteins to their target sequence (Truernit *et al.*, 2006). These proteins have a broad range of expression pattern, however KNAT3/4/5/7 show cell type specific expression in roots affecting cell proliferation, elongation and differentiation. Although the mechanisms involved are largely unknown (Truernit *et al.*, 2006; Li *et al.*, 2012). It is possible to speculate KNATs relation with stress response since the root is the primary organ that detects changes in soil conditions such as water deficits or high levels of salt (Choi *et al.*, 2014; Ghosh and Xu, 2014; Kaneko *et al.*, 2015). Truernit and Haseloff showed that the presence of ethylene increased the activity of KNAT5 promoter and that the cytokinins decreased the activity of KNAT3 promoter, while KNAT4 promoter seems not to be affected by these hormone treatment (Truernit and Haseloff, 2007). The KNAT7 seems to be involved in the regulation of the secondary wall biosynthesis in coordination with TFs of the NAC and MYB classes (Li *et al.*, 2012) namely MYB75 that interact with KNAT7 to regulate secondary cell wall formation in Arabidopsis inflorescence, stem vasculature and in seed coat (Bhargava *et al.*, 2013). The physiological relevance of the interaction between SnRK1β1 and members of the KNAT family has not been addressed, but this interaction might be important for the orchestrate coordination of the response to energy stress and development process like root development.

Glutamate synthase 1 (GLU1) is the other protein identified in the screen and for which the confirmation of the interaction was carried out. Since GLU1 present a high sequence similarity with Glutamate synthase 2 (GLU2) the interaction of this protein with SnRK1β1 was also studied. Interestingly, in Y2H assay, GLU2 showed a stronger interaction with SnRK1β1 than GLU1 (figure 6). Both GLU1 and GLU2 are Ferredoxin-dependent enzymes (Fd-GOGAT 1/2) (Coschigano *et al.*, 1998). Fd-GOGAT is the most predominant form of glutamate synthase in *Arabidopsis* and most likely is essential for photorespiration since knock down of Fd-GOGAT leads to a chlorotic and lethal phenotype under photorespiration conditions (Coschigano *et al.*, 1998; Kissen *et al.*, 2010; Aliyev, 2012). Moreover, in Fd-GOGAT mutants, several genes related to stimuli and

stress response including TFs such as zinc finger proteins, MYBs and bHLH, DREB2C/DREB2H are up-regulated (Kissen *et al.*, 2010). GLU1 and GLU2 shows different expression patterns. GLU1 mRNA is more present in leaves while GLU2 mRNA accumulates in roots and is likely involved in primary nitrogen assimilation (Coschigano *et al.*, 1998). These proteins play an important role in nitrate uptake and its assimilation (Debouba *et al.*, 2006). Noteworthy, some serine/threonine residues of GLU1 have already been identified as phosphorylated (PhosPhAt 4.0 database) and one of these residues (serine) is located in a putative SnRK1 recognition sequence ([hydrophobic]X[R/K]XX[S/T]XXX[hydrophobic]) (Figure 1 in Appendix 3)(Vlad *et al.*, 2008). This specific pattern was discovered in GLU1 using the tool "Patmatch" in *The Arabidopsis Information Resource* (TAIR). Thus, this evidence shows a possible interaction not only with β-subunit but also with SnRK1.1. Interestingly, one of the few known substrates of SnRK1 is nitrate reductase (Douglas *et al.*, 1997) an enzyme also involved in the nitrogen cycle. The results obtained might indicate that GLU1 and GLU2 are also likely substrates of SnRK1 suggesting that nitrate metabolism, which must be coordinated for optimized growth, is regulated by SnRK1 at multiple levels.

To further characterize the interaction between these identified proteins and the SnRK1 complex the possible interaction between these proteins and SnRK1.1 was also addressed. After 2 days at 30 °C no growth was verified (figure 7). However, after 7 days more, at room temperature some of the yeast colonies began to growth likely evidencing a very weak interaction with the α-subunit. However, even after this long incubation period U1A, U2B", GLU1 and GLU2 presented no evidences of interaction with the α-subunit (figure 8). The interaction between a kinase and its substrate is frequently labile, so it would be worth to perform the Y2H assay in less stringent conditions.

# 5. Future perspectives

It is well known the importance of the SnRK1 complex in the regulation of energy homeostasis, growth and development, and in the response to biotic and/or abiotic stresses. However, the subjacent molecular mechanisms are largely unknown.

The results obtained in this study uncovered several novel putative interactors of SnRK1 and suggests a novel molecular link between SnRK1 and RNA splicing (U1A and U2B"), development (KNAT proteins) and nitrogen metabolism (GLU1 and GLU2).

The identified interaction need to be further confirmed using other techniques either *in vitro* or *in vivo* such as pull down and Bimolecular Fluorescence Complementation (BiFC) analysis.

It would also be interesting to confirm that the identified putative interactors are real substrates of SnRK1, using in vitro phosphorylation assays with recombinant protein and/or accessing the phosphorylation state of those proteins *in vivo*.

Most importantly the physiological relevance of the identified interactions should be addressed. This could done using mutants affected in the expression of those proteins and/or of SnRK1 complex subunits, or for example by transient expression assays in protoplast.

In this screen several other proteins whose interaction with SnRK1 is potentially worth to analyze in further detail have been identified - among them we could highlight a heat shock protein (HSP70-2) and two proteins involved in the ubiquitination process, the UBC18 protein and the DAFL1 protein (AT3G10910).

# References

**Akpinar, B.A., Avsar, B., Lucas, S.J., and Budak, H.** (2012). Plant abiotic stress signaling.

**Aliyev, J.A.** (2012). Photosynthesis, photorespiration and productivity of wheat and soybean genotypes. Physiologia Plantarum 145, 369-383.

**Avila, J., Gregory, O.G., Su, D., Deeter, T.A., Chen, S., Silva-Sanchez, C., Xu, S., Martin, G.B., and Devarenne, T.P.** (2012). The beta-Subunit of the SnRK1 Complex Is Phosphorylated by the Plant Cell Death Suppressor Adi3. Plant Physiology 159, 1277-1290.

**Avila-Castaneda, A., Gutierrez-Granados, N., Ruiz-Gayosso, A., Sosa-Peinado, A., Martinez-Barajas, E., and Coello, P.** (2014). Structural and functional basis for starch binding in the SnRK1 subunits AKIN beta 2 and AKIN beta gamma. Frontiers in Plant Science 5.

**Baena-Gonzalez, E.** (2010). Energy Signaling in the Regulation of Gene Expression during Stress. Molecular Plant 3, 300-313.

**Baena-Gonzalez, E., and Sheen, J.** (2008). Convergent energy and stress signaling. Trends in Plant Science 13, 474-482.

**Baena-Gonzalez, E., Rolland, F., Thevelein, J.M., and Sheen, J.** (2007). A central integrator of transcription networks in plant stress and energy signalling. Nature 448, 938-U910.

**Beg, Z.H., Allmann, D.W., and Gibson, D.M.** (1973). Modulation of 3-hydroxy-3-methylglutaryl coenzyme a reductase-activity with camp and with protein fractions of rat-liver cytosol. Biochemical and Biophysical Research Communications 54, 1362-1369.

**Bhargava, A., Ahad, A., Wang, S.C., Mansfield, S.D., Haughn, G.W., Douglas, C.J., and Ellis, B.E.** (2013). The interacting MYB75 and KNAT7 transcription factors modulate secondary cell wall deposition both in stems and seed coat in *Arabidopsis*. Planta 237, 1199-1211.

**Bolingue, W., Rosnoblet, C., Leprince, O., Vu, B.L., Aubry, C., and Buitink, J.** (2010). The MtSNF4b subunit of the sucrose non-fermenting-related kinase complex connects after-ripening and constitutive defense responses in seeds of *Medicago truncatula*. Plant Journal 61, 792-803.

**Bouly, J.P., Gissot, L., Lessard, P., Kreis, M., and Thomas, M.** (1999). Arabidopsis *thaliana* proteins related to the yeast SIP and SNF4 interact with AKIN alpha 1, an SNF1-like protein kinase. Plant Journal 18, 541-550.

**Bradford, K.J., Downie, A.B., Gee, O.H., Alvarado, V., Yang, H., and Dahal, P.** (2003). Abscisic acid and gibberellin differentially regulate expression of genes of the SNF1-related kinase complex in tomato seeds. Plant Physiology 132, 1560-1576.

**Brodsky, A.S., and Silver, P.A.** (2000). Pre-mRNA processing factors are required for nuclear export. RNA-a Publication of the RNA Society 6, 1737-1749.

**Cabello, J.V., Lodeyro, A.F., and Zurbriggen, M.D.** (2014). Novel perspectives for the engineering of abiotic stress tolerance in plants. Current Opinion in Biotechnology 26, 62-70.

**Carlson, C.A., and Kim, K.** (1973). Regulation of hepatic acetyl coenzyme-a carboxylase by phosphorylation and dephosphorylation. Journal of Biological Chemistry 248, 378-380.

**Carlson, M., Osmond, B.C., and Botstein, D.** (1981). Mutants of yeast defective in sucrose utilization. Genetics 98, 25-40.

**Celenza, J.L., and Carlson, M.** (1984). Structure and expression of the snf1 gene of *saccharomyces cerevisiae*. Molecular and Cellular Biology 4, 54-60.

**Celenza, J.L., and Carlson, M.** (1989). Mutational analysis of the *Saccharomyces cerevisiae* snf1 protein-kinase and evidence for functional interaction with the snf4 protein. Molecular and Cellular Biology 9, 5034-5044.

**Cho, Y.-H., Hong, J.-W., Kim, E.-C., and Yoo, S.-D.** (2012). Regulatory Functions of SnRK1 in Stress-Responsive Gene Expression and in Plant Growth and Development. Plant Physiology 158, 1955-1964.

**Choi, W.G., Toyota, M., Kim, S.H., Hilleary, R., and Gilroy, S.** (2014). Salt stress-induced Ca2+ waves are associated with rapid, long-distance root-to-shoot signaling in plants. Proceedings of the National Academy of Sciences of the United States of America 111, 6497-6502.

**Clark, H., Carling, D., and Saggerson, D.** (2004). Covalent activation of heart AMP-activated protein kinase in response to physiological concentrations of long-chain fatty acids. European Journal of Biochemistry 271, 2215-2224.

**Coello, P., and Martinez-Barajas, E.** (2014). SnRK1 is differentially regulated in the cotyledon and embryo axe of bean (*Phaseolus vulgaris L*) seeds. Plant Physiology and Biochemistry 80, 153-159.

**Confraria, A., Martinho, C., Elias, A., Rubio-Somoza, I., and Baena-Gonzalez, E.** (2013). miRNAs mediate SnRK1-dependent energy signaling in *Arabidopsis*. Frontiers in Plant Science 4.

**Coschigano, K.T., Melo-Oliveira, R., Lim, J., and Coruzzi, G.M.** (1998). *Arabidopsis* gls mutants and distinct Fd-GOGAT genes: Implications for photorespiration and primary nitrogen assimilation. Plant Cell 10, 741-752.

**Crozet, P., Jammes, F., Valot, B., Ambard-Bretteville, F., Nessler, S., Hodges, M., Vidal, J., and Thomas, M.** (2010). Cross-phosphorylation between *Arabidopsis thaliana* Sucrose Non-fermenting 1-related Protein Kinase 1 (AtSnRK1) and Its Activating Kinase (AtSnAK) Determines Their Catalytic Activities. Journal of Biological Chemistry 285, 12071-12077.

**Crozet, P., Margalha, L., Confraria, A., Rodrigues, A., Martinho, C., Adamo, M., Elias, C.A., and Baena-Gonzalez, E.** (2014). Mechanisms of regulation of SNF1/AMPK/SnRK1 protein kinases. Frontiers in Plant Science 5.

**Danquah, A., de Zelicourt, A., Colcombet, J., and Hirt, H.** (2014). The role of ABA and MAPK signaling pathways in plant abiotic stress responses. Biotechnology Advances 32, 40-52.

**Debouba, M., Gouia, H., Valadier, M.H., Ghorbel, M.H., and Suzuki, A.** (2006). Salinity-induced tissue-specific diurnal changes in nitrogen assimilatory enzymes in tomato seedlings grown under high or low nitrate medium. Plant Physiology and Biochemistry 44, 409-419.

**Douglas, P., Pigaglio, E., Ferrer, A., Halford, N.G., and MacKintosh, C.** (1997). Three spinach leaf nitrate reductase-3-hydroxy-3-methylglutaryl-CoA reductase kinases that are regulated by reversible phosphorylation and/or Ca2+ ions. Biochemical Journal 325, 101-109.

**Duque, P.** (2011). A role for SR proteins in plant stress responses. Plant signaling & behavior 6, 49-54.

**Durek, P., Schmidt, R., Heazlewood, J.L., Jones, A., MacLean, D., Nagel, A., Kersten, B., and Schulze, W.X.** (2010). PhosPhAt: the *Arabidopsis thaliana* phosphorylation site database. An update. Nucleic Acids Research 38, D828-D834.

**Emanuelle, S., Hossain, M.I., Moller, I.E., Pedersen, H.L., van de Meene, A.M.L., Doblin, M.S., Koay, A., Oakhill, J.S., Scott, J.W., Willats, W.G.T., Kemp, B.E., Bacic, A., Gooley, P.R., and Stapleton, D.I.** (2015). SnRK1 from *Arabidopsis thaliana* is an atypical AMPK. Plant Journal 82, 183-192.

**Fang, L., Hou, X., Yen, L., Lee, C., Liu, L., Yan, X., and Yu, H.** (2011). AtPV42a and AtPV42b Redundantly Regulate Reproductive Development in *Arabidopsis thaliana*. Plos One 6.

**Forment, J., Naranjo, M.A., Roldan, M., Serrano, R., and Vicente, O.** (2002). Expression of *Arabidopsis* SR-like splicing proteins confers salt tolerance to yeast and transgenic plants. Plant Journal 30, 511-519.

**Fragoso, S., Espindola, L., Paez-Valencia, J., Gamboa, A., Camacho, Y., Martinez-Barajas, E., and Coello, P.** (2009). SnRK1 Isoforms AKIN10 and AKIN11 Are Differentially Regulated in *Arabidopsis* Plants under Phosphate Starvation. Plant Physiology 149, 1906-1916.

**Fujiki, Y., Ito, M., Nishida, I., and Watanabe, A.** (2000). Multiple signaling pathways in gene expression during sugar starvation. Pharmacological analysis of din gene expression in suspension-cultured cells of *Arabidopsis*. Plant Physiology 124, 1139-1147.

**Fujiki, Y., Yoshikawa, Y., Sato, T., Inada, N., Ito, M., Nishida, I., and Watanabe, A.** (2001). Dark-inducible genes from *Arabidopsis thaliana* are associated with leaf senescence and repressed by sugars. Physiologia Plantarum 111, 345-352.

**Fujita, Y., Nakashima, K., Yoshida, T., Katagiri, T., Kidokoro, S., Kanamori, N., Umezawa, T., Fujita, M., Maruyama, K., Ishiyama, K., Kobayashi, M., Nakasone, S., Yamada, K., Ito, T., Shinozaki, K., and Yamaguchi-Shinozaki, K.** (2009). Three SnRK2 Protein Kinases are the Main Positive Regulators of Abscisic Acid Signaling in Response to Water Stress in *Arabidopsis*. Plant and Cell Physiology 50, 2123-2132.

**Gehring, W.J.** (1987). Homeo boxes in the study of development. Science 236, 1245-1252.

**Ghillebert, R., Swinnen, E., Wen, J., Vandesteene, L., Ramon, M., Norga, K., Rolland, F., and Winderickx, J.** (2011). The AMPK/SNF1/SnRK1 fuel gauge and energy regulator: structure, function and regulation. Febs Journal 278, 3978-3990.

**Ghosh, D., and Xu, J.** (2014). Abiotic stress responses in plant roots: a proteomics perspective. Frontiers in Plant Science 5, 13.

**Gietz, R.D., TriggsRaine, B., Robbins, A., Graham, K.C., and Woods, R.A.** (1997). Identification of proteins that interact with a protein of interest: Applications of the yeast two-hybrid system. Molecular and Cellular Biochemistry 172, 67-79.

**Gissot, L., Polge, C., Bouly, J.P., Lemaitre, T., Kreis, M., and Thomas, M.** (2004). AKIN beta 3, a plant specific SnRK1 protein, is lacking domains present in yeast and mammals non-catalytic beta-subunits. Plant Molecular Biology 56, 747-759.

**Gissot, L., Polge, C., Jossier, M., Girin, T., Bouly, J.-P., Kreis, M., and Thomas, M.** (2006). AKIN beta gamma contributes to SnRK1 heterotrimeric complexes and interacts with two proteins implicated in plant pathogen resistance through its KIS/GBD sequence. Plant Physiology 142, 931-944.

**Guo, X.-H., Deng, K.-Q., Wang, J., Yu, D.-S., Zhao, Q., and Liu, X.-M.** (2010). Mutational analysis of *Arabidopsis* PP2CA2 involved in abscisic acid signal transduction. Molecular Biology Reports 37, 763-769.

**Habenicht, A.** (1997). The non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase: Biochemistry, structure, occurrence and evolution. Biological Chemistry 378, 1413-1419.

**Halford, N.G., and Hardie, D.G.** (1998). SNF1-related protein kinases: global regulators of carbon metabolism in plants? Plant Molecular Biology 37, 735-748.

**Halford, N.G., Hey, S., Jhurreea, D., Laurie, S., McKibbin, R.S., Paul, M., and Zhang, Y.H.** (2003). Metabolic signalling and carbon partitioning: role of Snf1-related (SnRK1) protein kinase. Journal of Experimental Botany 54, 467-475.

**Hanson, J., and Smeekens, S.** (2009). Sugar perception and signaling - an update. Current Opinion in Plant Biology 12, 562-567.

**Hardie, D.G.** (2007). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nature Reviews Molecular Cell Biology 8, 774-785.

**Hardie, D.G.** (2011). Sensing of energy and nutrients by AMP-activated protein kinase. American Journal of Clinical Nutrition 93, 891S-896S.

**Hardie, D.G., Carling, D., and Carlson, M.** (1998). The AMP-activated/SNF1 protein kinase subfamily: Metabolic sensors of the eukaryotic cell? Annual Review of Biochemistry 67, 821-855.

**Hardie, D.G., Carling, D., and Gamblin, S.J.** (2011). AMP-activated protein kinase: also regulated by ADP? Trends in Biochemical Sciences 36, 470-477.

**Hardie, D.G., Ross, F.A., and Hawley, S.A.** (2012). AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nature Reviews Molecular Cell Biology 13, 251-262.

**Harmer, S.L., Hogenesch, L.B., Straume, M., Chang, H.S., Han, B., Zhu, T., Wang, X., Kreps, J.A., and Kay, S.A.** (2000). Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science 290, 2110-2113.

**Harthill, J.E., Meek, S.E.M., Morrice, N., Peggie, M.W., Borch, J., Wong, B.H.C., and MacKintosh, C.** (2006). Phosphorylation and 14-3-3 binding of *Arabidopsis* trehalose-phosphate synthase 5 in response to 2-deoxyglucose. Plant Journal 47, 211-223.

**Hawley, S.A., Davison, M., Woods, A., Davies, S.P., Beri, R.K., Carling, D., and Hardie, D.G.** (1996). Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. Journal of Biological Chemistry 271, 27879-27887.

**Heazlewood, J.L., Durek, P., Hummel, J., Selbig, J., Weckwerth, W., Walther, D., and Schulze, W.X.** (2008). PhosPhAt: a database of phosphorylation sites in *Arabidopsis thaliana* and a plant-specific phosphorylation site predictor. Nucleic Acids Research 36, D1015-D1021.

**Hedbacker, K., and Carlson, M.** (2008). SNF1/AMPK pathways in yeast. Frontiers in Bioscience-Landmark 13, 2408-2420.

**Hedbacker, K., Hong, S.P., and Carlson, M.** (2004). Pak1 protein kinase regulates activation and nuclear localization of Snf1-Gal83 protein kinase. Molecular and Cellular Biology 24, 8255-8263.

**Hedges, D., Proft, M., and Entian, K.D.** (1995). CAT8, a new zinc cluster-encoding gene necessary for derepression of gluconeogenic enzymes in the yeast *Saccharomyces cerevisiae*. Molecular and Cellular Biology 15, 1915-1922.

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

**Hirel, B., Tetu, T., Lea, P.J., and Dubois, F.** (2011). Improving Nitrogen Use Efficiency in Crops for Sustainable Agriculture. Sustainability 3, 1452-1485.

**Hoffman, C.S., and Winston, F.** (1987). A 10-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene 57, 267-272.

**Hong, S.P., Leiper, F.C., Woods, A., Carling, D., and Carlson, M.** (2003). Activation of yeast SNF1 and mammalian AMP-activated protein kinase by upstream kinases. Proceedings of the National Academy of Sciences of the United States of America 100, 8839-8843.

**Hrabak, E.M., Chan, C.W.M., Gribskov, M., Harper, J.F., Choi, J.H., Halford, N., Kudla, J., Luan, S., Nimmo, H.G., Sussman, M.R., Thomas, M., Walker-Simmons, K., Zhu, J.K., and Harmon, A.C.** (2003). The *Arabidopsis* CDPK-SnRK superfamily of protein kinases. Plant Physiology 132, 666-680.

**Huang, G.T., Ma, S.L., Bai, L.P., Zhang, L., Ma, H., Jia, P., Liu, J., Zhong, M., and Guo, Z.F.** (2012). Signal transduction during cold, salt, and drought stresses in plants. Molecular Biology Reports **39,** 969-987.

**Jones, R.G., Plas, D.R., Kubek, S., Buzzai, M., Mu, J., Xu, Y., Birnbaum, M.J., and Thompson, C.B.** (2005). AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. Molecular Cell 18, 283-293.

**Jossier, M., Bouly, J.-P., Meimoun, P., Arjmand, A., Lessard, P., Hawley, S., Grahame Hardie, D., and Thomas, M.** (2009). SnRK1 (SNF1-related kinase 1) has a central role in sugar and ABA signalling in *Arabidopsis thaliana*. Plant Journal 59, 316-328.

**Kaneko, T., Horie, T., Nakahara, Y., Tsuji, N., Shibasaka, M., and Katsuhara, M.** (2015). Dynamic Regulation of the Root Hydraulic Conductivity of Barley Plants in Response to Salinity/Osmotic Stress. Plant and Cell Physiology 56, 875-882.

**Kangasjarvi, S., Neukermans, J., Li, S.C., Aro, E.M., and Noctor, G.** (2012). Photosynthesis, photorespiration, and light signalling in defence responses. Journal of Experimental Botany 63, 1619-1636.

**Kim, J.Y., Yuan, Z., and Jackson, D.** (2003). Developmental regulation and significance of KNOX protein trafficking in *Arabidopsis.* Development 130, 4351-4362.

**Kim, M.D., Hong, S.P., and Carlson, M.** (2005). Role of Tos3, a Snf1 protein kinase kinase, during growth of *Saccharomyces cerevisiae* on non-fermentable carbon sources. Eukaryotic Cell 4, 861-866.

**Kissen, R., Winge, P., Tran, D.H.T., Jorstad, T.S., Storseth, T.R., Christensen, T., and Bones, A.M**. (2010). Transcriptional profiling of an Fd-GOGAT1/GLU1 mutant in *Arabidopsis thaliana* reveals a multiple stress response and extensive reprogramming of the transcriptome. Bmc Genomics 11.

**Laurie, S., and Halford, N.G.** (2001). The role of protein kinases in the regulation of plant growth and development. Plant Growth Regulation 34, 253-265.

**Law, M.J., Rice, A.J., Lin, P., and Laird-Offringa, I.A.** (2006). The role of RNA structure in the interaction of U1A protein with U1 hairpin II RNA. RNA-a Publication of the RNA Society 12, 1168-1178.

**Lee, J.H., Koh, H., Kim, M., Kim, Y., Lee, S.Y., Karess, R.E., Lee, S.H., Shong, M., Kim, J.M., Kim, J., and Chung, J.K.** (2007). Energy-dependent regulation of cell structure by AMP-activated protein kinase. Nature 447, 1017-U1019.

**Lee, S.C., Mustroph, A., Sasidharan, R., Vashisht, D., Pedersen, O., Oosumi, T., Voesenek, L.A.C.J., and Bailey-Serres, J.** (2011). Molecular characterization of the submergence response of the *Arabidopsis thaliana* ecotype Columbia. New Phytologist 190, 457-471.

**Lesage, P., Yang, X.L., and Carlson, M.** (1996). Yeast SNF1 protein kinase interacts with SIP4, a C-6 zinc cluster transcriptional activator: A new role for SNF1 in the glucose response. Molecular and Cellular Biology 16, 1921-1928.

**Li, E.Y., Bhargava, A., Qiang, W.Y., Friedmann, M.C., Forneris, N., Savidge, R.A., Johnson, L.A., Mansfield, S.D., Ellis, B.E., and Douglas, C.J.** (2012). The Class II KNOX gene KNAT7 negatively regulates secondary wall formation in *Arabidopsis* and is functionally conserved in *Populus*. New Phytologist 194, 102-115.

**Li, G., Peng, F., Zhang, L., Shi, X., and Wang, Z.** (2010). Cloning and characterization of a SnRK1-encoding gene from *Malus hupehensis Rehd*. and heterologous expression in tomato. Molecular Biology Reports 37, 947-954.

**Li, X.-F., Li, Y.-J., An, Y.-H., Xiong, L.-J., Shao, X.-H., Wang, Y., and Sun, Y.** (2009). AKIN beta 1 is involved in the Regulation of Nitrogen Metabolism and Sugar Signaling in *Arabidopsis*. Journal of Integrative Plant Biology 51, 513-520.

**Liang, J., Xu, Z.-X., Ding, Z., Lu, Y., Yu, Q., Werle, K.D., Zhou, G., Park, Y.-Y., Peng, G., Gambello, M.J., and Mills, G.B.** (2015). Myristoylation confers non-canonical AMPK functions in autophagy selectivity and mitochondrial surveillance. Nature communications 6, 7926.

**Liang, J.Y., and Mills, G.B.** (2013). AMPK: A Contextual Oncogene or Tumor Suppressor? Cancer Research 73, 2929-2935.

**Liang, J.Y., Shao, S.H., Xu, Z.X., Hennessy, B., Ding, Z.Y., Larrea, M., Kondo, S., Dumont, D.J., Gutterman, J.U., Walker, C.L., Slingerland, J.M., and Mills, G.B.**

(2007). The energy sensing LKB1-AMPK pathway regulates p27 (kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. Nature Cell Biology 9, 218-U125.

**Lin, S.S., Manchester, J.K., and Gordon, J.I.** (2003). Sip2, an N-myristoylated beta subunit of Snf1 kinase, regulates aging in *Saccharomyces cerevisiae* by affecting cellular histone kinase activity, recombination at rDNA loci, and silencing. Journal of Biological Chemistry 278, 13390-13397.

**Lopez-Paz, C., Vilela, B., Riera, M., Pages, M., and Lumbreras, V.** (2009). Maize AKIN beta gamma dimerizes through the KIS/CBM domain and assembles into SnRK1 complexes. Febs Letters 583, 1887-1894.

**Lovas, A., Bimbo, A., Szabo, L., and Banfalvi, Z.** (2003). Antisense repression of StubGAL83 affects root and tuber development in potato. Plant Journal 33, 139-147.

**Lu, C.A., Lin, C.C., Lee, K.W., Chen, J.L., Huang, L.F., Ho, S.L., Liu, H.J., Hsing, Y.I., and Yu, S.M.** (2007). The SnRK1A protein kinase plays a key role in sugar signaling during germination and seedling growth of rice. Plant Cell 19, 2484-2499.

**Ludin, K., Jiang, R., and Carlson, M.** (1998). Glucose-regulated interaction of a regulatory subunit of protein phosphatase 1 with the Snf1 protein kinase in *Saccharomyces cerevisiae*. Proceedings of the National Academy of Sciences of the United States of America 95, 6245-6250.

**Lumbreras, V., Alba, M.M., Kleinow, T., Koncz, C., and Pages, M.** (2001). Domain fusion between SNF1-related kinase subunits during plant evolution. Embo Reports 2, 55-60.

**Mayer, F.V., Heath, R., Underwood, E., Sanders, M.J., Carmena, D., McCartney, R.R., Leiper, F.C., Xiao, B., Jing, C., Walker, P.A., Haire, L.F., Ogrodowicz, R., Martin, S.R., Schmidt, M.C., Gamblin, S.J., and Carling, D.** (2011). ADP Regulates SNF1, the *Saccharomyces cerevisiae* Homolog of AMP-Activated Protein Kinase. Cell Metabolism 14, 707-714.

**McCartney, R.R., and Schmidt, M.C.** (2001). Regulation of Snf1 kinase - Activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. Journal of Biological Chemistry 276, 36460-36466.

**McKibbin, R.S., Muttucumaru, N., Paul, M.J., Powers, S.J., Burrell, M.M., Coates, S., Purcell, P.C., Tiessen, A., Geigenberger, P., and Halford, N.G.** (2006). Production

of high-starch, low-glucose potatoes through over-expression of the metabolic regulator SnRK1. Plant Biotechnology Journal 4, 409-418.

**Miyashita, Y., and Good, A.G.** (2008). NAD(H)-dependent glutamate dehydrogenase is essential for the survival of *Arabidopsis thaliana* during dark-induced carbon starvation. Journal of Experimental Botany 59, 667-680.

**Nath, N., McCartney, R.R., and Schmidt, M.C.** (2003). Yeast Pak1 kinase associates with and activates Snf1. Molecular and Cellular Biology 23, 3909-3917.

**Nietzsche, M., Schiessl, I., and Boernke, F.** (2014). The complex becomes more complex: protein-protein interactions of SnRK1 with DUF581 family proteins provide a framework for cell and stimulus type-specific SnRK1 signaling in plants. Frontiers in Plant Science 5.

**Nunes, C., O'Hara, L.E., Primavesi, L.F., Delatte, T.L., Schluepmann, H., Somsen, G.W., Silva, A.B., Fevereiro, P.S., Wingler, A., and Paul, M.J.** (2013b). The Trehalose 6-Phosphate/SnRK1 Signaling Pathway Primes Growth Recovery following Relief of Sink Limitation. Plant Physiology 162, 1720-1732.

**Nunes, C., Primavesi, L.F., Patel, M.K., Martinez-Barajas, E., Powers, S.J., Sagar, R., Fevereiro, P.S., Davis, B.G., and Paul, M.J.** (2013a). Inhibition of SnRK1 by metabolites: Tissue-dependent effects and cooperative inhibition by glucose 1-phosphate in combination with trehalose 6-phosphate. Plant Physiology and Biochemistry 63, 89-98.

**Oakhill, J.S., Chen, Z.P., Scott, J.W., Steel, R., Castelli, L.A., Ling, N.M., Macaulay, S.L., and Kemp, B.E.** (2010). Beta-Subunit myristoylation is the gatekeeper for initiating metabolic stress sensing by AMP-activated protein kinase (AMPK). Proceedings of the National Academy of Sciences of the United States of America 107, 19237-19241.

**Oakhill, J.S., Steel, R., Chen, Z.-P., Scott, J.W., Ling, N., Tam, S., and Kemp, B.E.** (2011). AMPK Is a Direct Adenylate Charge-Regulated Protein Kinase. Science 332, 1433-1435.

**Pierre, M., Traverso, J.A., Boisson, B., Domenichini, S., Bouchez, D., Giglione, C., and Meinnel, T.** (2007). N-myristoylation regulates the SnRK1 pathway in *Arabidopsis*. Plant Cell 19, 2804-2821.

**Polekhina, G., Gupta, A., van Denderen, B.J.W., Fell, S.C., Kemp, B.E., Stapleton, D., and Parker, M.W.** (2005). Structural basis for glycogen recognition by AMP-activated protein kinase. Structure 13, 1453-1462.

**Polge, C., and Thomas, M.** (2007). SNF1/AMPK/SnRK1 kinases, global regulators at the heart of energy control? Trends in Plant Science 12, 20-28.

**Polge, C., Jossier, M., Crozet, P., Gissot, L., and Thomas, M.** (2008). beta-Subunits of the SnRK1 Complexes Share a Common Ancestral Function Together with Expression and Function Specificities; Physical Interaction with Nitrate Reductase Specifically Occurs via AKIN beta 1-Subunit. Plant Physiology 148, 1570-1582.

**Radchuk, R., Emery, R.J.N., Weier, D., Vigeolas, H., Geigenberger, P., Lunn, J.E., Feil, R., Weschke, W., and Weber, H.** (2010). Sucrose non-fermenting kinase 1 (SnRK1) coordinates metabolic and hormonal signals during pea cotyledon growth and differentiation. Plant Journal 61, 324-338.

**Radchuk, R., Radchuk, V., Weschke, W., Borisjuk, L., and Weber, H.** (2006). Repressing the expression of the sucrose nonfermenting-1-related protein kinase gene in pea embryo causes pleiotropic defects of maturation similar to an abscisic acid-insensitive phenotype. Plant Physiology 140, 263-278.

**Ramon, M., Rolland, F., and Sheen, J.** (2008). Sugar sensing and signaling. The *Arabidopsis* book / American Society of Plant Biologists 6, e0117-e0117.

**Ramon, M., Ruelens, P., Li, Y., Sheen, J., Geuten, K., and Rolland, F.** (2013). The hybrid Four-CBS-Domain KIN subunit functions as the canonical subunit of the plant energy sensor SnRK1. Plant Journal 75, 11-25.

**Rodrigues, A., Adamo, M., Crozet, P., Margalha, L., Confraria, A., Martinho, C., Elias, A., Rabissi, A., Lumbreras, V., Gonzalez-Guzman, M., Antoni, R., Rodriguez, P.L., and Baena-Gonzalez, E.** (2013). ABI1 and PP2CA Phosphatases Are Negative Regulators of Snf1-Related Protein Kinase1 Signaling in *Arabidopsis*. Plant Cell 25, 3871-3884.

**Rolland, F., Baena-Gonzalez, E., and Sheen, J.** (2006). Sugar sensing and signaling in plants: Conserved and novel mechanisms. Annual Review of Plant Biology 57, 675-709.

**Rolland, F., Moore, B., and Sheen, J.** (2002). Sugar sensing and signaling in plants. Plant Cell 14, S185-S205.

**Rosa, M., Prado, C., Podazza, G., Interdonato, R., Gonzalez, J.A., Hilal, M., and Prado, F.E.** (2009). Soluble sugars - metabolism, sensing and abiotic stress: a complex network in the life of plants. Plant signaling & behavior 4, 388-393.

**Rosnoblet, C., Aubry, C., Leprince, O., Vu, B.L., Rogniaux, H., and Buitink, J.** (2007). The regulatory gamma subunit SNF4b of the sucrose non-fermenting-related kinase complex is involved in longevity and stachyose accumulation during maturation of *Medicago truncatula* seeds. Plant Journal 51, 47-59.

**Ruepp, A., Zollner, A., Maier, D., Albermann, K., Hani, J., Mokrejs, M., Tetko, I., Guldener, U., Mannhaupt, G., Munsterkotter, M., and Mewes, H.W.** (2004). The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. Nucleic Acids Research 32, 5539-5545.

**Ruiz, A., Xu, X.J., and Carlson, M.** (2011). Roles of two protein phosphatases, Reg1-Glc7 and Sit4, and glycogen synthesis in regulation of SNF1 protein kinase. Proceedings of the National Academy of Sciences of the United States of America 108, 6349-6354.

**Rutjens, B., Bao, D.P., van Eck-Stouten, E., Brand, M., Smeekens, S., and Proveniers, M.** (2009). Shoot apical meristem function in *Arabidopsis* requires the combined activities of three BEL1-like homeodomain proteins. Plant Journal 58, 641-654.

**Sakamoto, T., Kamiya, N., Ueguchi-Tanaka, M., Iwahori, S., and Matsuoka, M.** (2001). KNOX homeodomain protein directly suppresses the expression of a gibberellin biosynthetic gene in the tobacco shoot apical meristem. Genes & Development 15, 581-590.

**Sanders, D., Brownlee, C., and Harper, J.F.** (1999). Communicating with calcium. Plant Cell **11,** 691-706.

**Santiago, J., Rodrigues, A., Saez, A., Rubio, S., Antoni, R., Dupeux, F., Park, S.-Y., Marquez, J.A., Cutler, S.R., and Rodriguez, P.L.** (2009). Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs. Plant Journal 60, 575-588.

**Schmidt, M.C., and McCartney, R.R.** (2000). Beta-subunits of Snf1 kinase are required for kinase function and substrate definition. Embo Journal 19, 4936-4943.

**Schwachtje, J., Minchin, P.E.H., Jahnke, S., van Dongen, J.T., Schittko, U., and Baldwin, I.T.** (2006). SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. Proceedings of the National Academy of Sciences of the United States of America 103, 12935-12940.

**Scofield, S., Dewitte, W., and Murray, J.A.** (2008). A model for *Arabidopsis* class-1 KNOX gene function. Plant signaling & behavior 3, 257-259.

**Serikawa, K.A., MartinezLaborda, A., and Zambryski, P.** (1996). Three knotted1-like homeobox genes in *Arabidopsis*. Plant Molecular Biology 32, 673-683.

**Serrano, R., Mulet, J.M., Rios, G., Marquez, J.A., de Larrinoa, I.F., Leube, M.P., Mendizabal, I., Pascual-Ahuir, A., Proft, M., Ros, R., and Montesinos, C.** (1999). A glimpse of the mechanisms of ion homeostasis during salt stress. Journal of Experimental Botany 50, 1023-1036.

**Sheen, J.** (1998). Mutational analysis of protein phosphatase 2C involved in abscisic acid signal transduction in higher plants. Proceedings of the National Academy of Sciences of the United States of America 95, 975-980.

**Shen, W., Reyes, M.I., and Hanley-Bowdoin, L.** (2009). *Arabidopsis* Protein Kinases GRIK1 and GRIK2 Specifically Activate SnRK1 by Phosphorylating Its Activation Loop. Plant Physiology 150, 996-1005.

**Simpson, G.G., Clark, G.P., Rothnie, H.M., Boelens, W., Vanvenrooij, W., and Brown, J.W.S.** (1995). Molecular characterization of the spliceosomal proteins U1A and U2B" from higher-plants. Embo Journal 14, 4540-4550.

**Spasic, M.R., Callaerts, P., and Norga, K.K.** (2008). Drosophila alicorn is a neuronal maintenance factor protecting against activity-induced retinal degeneration. Journal of Neuroscience 28, 6419-6429.

**Sugden, C., Crawford, R.M., Halford, N.G., and Hardie, D.G.** (1999a). Regulation of spinach SNF1-related (SnRK1) kinases by protein kinases and phosphatases is associated with phosphorylation of the T loop and is regulated by 5 '-AMP. Plant Journal 19, 433-439.

**Sugden, C., Donaghy, P.G., Halford, N.G., and Hardie, D.G.** (1999b). Two SNF1-Related protein kinases from spinach leaf phosphorylate and inactivate 3-hydroxy-3-methylglutaryl-coenzyme A reductase, nitrate reductase, and sucrose phosphate synthase in vitro. Plant Physiology 120, 257-274.

**Sutherland, C.M., Hawley, S.A., McCartney, R.R., Leech, A., Stark, M.J.R., Schmidt, M.C., and Hardie, D.G.** (2003). Elm1p is one of three upstream kinases for the *Saccharomyces cerevisiae* SNF1 complex. Current Biology 13, 1299-1305.

**Tahtiharju, S., and Palva, T.** (2001). Antisense inhibition of protein phosphatase 2C accelerates cold acclimation in *Arabidopsis thaliana*. Plant Journal 26, 461-470.

**Thelander, M., Olsson, T., and Ronne, H.** (2004). Snf1-related protein kinase 1 is needed for growth in a normal day-night light cycle. Embo Journal 23, 1900-1910.

**Thompson, A.R., Doelling, J.H., Suttangkakul, A., and Vierstra, R.D.** (2005). Autophagic nutrient recycling in *Arabidopsis* directed by the ATG8 and ATG12 conjugation pathways. Plant Physiology 138, 2097-2110.

**Tiessen, A., Prescha, K., Branscheid, A., Palacios, N., McKibbin, R., Halford, N.G., and Geigenberger, P.** (2003). Evidence that SNF1-related kinase and hexokinase are involved in separate sugar-signalling pathways modulating post-translational redox activation of ADP-glucose pyrophosphorylase in potato tubers. Plant Journal 35, 490-500.

**Traverso, J.A., Meinnel, T., and Giglione, C.** (2008). Expanded impact of protein N-myristoylation in plants. Plant signaling & behavior 3, 501-502.

**Treitel, M.A., Kuchin, S., and Carlson, M.** (1998). SNF1 protein kinase regulates phosphorylation of the mig1 repressor in *Saccharomyces cerevisiae*. Molecular and Cellular Biology 18, 6273-6280.

**Truernit, E., and Haseloff, J.** (2007). A Role for KNAT Class II Genes in Root Development. Plant signaling & behavior 2, 10-12.

**Truernit, E., Siemering, K.R., Hodge, S., Grbic, V., and Haseloff, J**. (2006). A map of KNAT gene expression in the *Arabidopsis* root. Plant Molecular Biology 60, 1-20.

**Tsai, A.Y.-L., and Gazzarrini, S.** (2012a). Overlapping and distinct roles of AKIN10 and FUSCA3 in ABA and sugar signaling during seed germination. Plant signaling & behavior 7, 1238-1242.

**Tsai, A.Y.-L., and Gazzarrini, S.** (2012b). AKIN10 and FUSCA3 interact to control lateral organ development and phase transitions in *Arabidopsis*. Plant Journal 69, 809-821.

**Tschape, J.A., Hammerschmied, C., Muhlig-Versen, M., Athenstaedt, K., Daum, G., and Kretzschmar, D.** (2002). The neurodegeneration mutant lochrig interferes with cholesterol homeostasis and Appl processing. Embo Journal 21, 6367-6376.

**Tu, J.L., and Carlson, M.** (1995). Reg1 binds to protein phosphatase type-1 and regulates glucose repression in *Saccharomyces cerevisiae*. Embo Journal 14, 5939-5946.

**Tuteja, N.** (2007). Abscisic Acid and abiotic stress signaling. Plant signaling & behavior 2, 135-138.

**Umezawa, T., Sugiyama, N., Mizoguchi, M., Hayashi, S., Myouga, F., Yamaguchi-Shinozaki, K., Ishihama, Y., Hirayama, T., and Shinozaki, K.** (2009). Type 2C protein

phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis.* Proceedings of the National Academy of Sciences of the United States of America 106, 17588-17593.

**Vanesa Piattoni, C., Martin Bustos, D., Adrian Guerrero, S., and Alvaro Iglesias, A.** (2011). Nonphosphorylating Glyceraldehyde-3-Phosphate Dehydrogenase Is Phosphorylated in Wheat Endosperm at Serine-404 by an SNF1-Related Protein Kinase Allosterically Inhibited by Ribose-5-Phosphate. Plant Physiology 156, 1337-1350.

**Vincent, O., Townley, R., Kuchin, S., and Carlson, M.** (2001). Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. Genes & Development 15, 1104-1114.

**Vlad, F., Turk, B.E., Peynot, P., Leung, J., and Merlot, S.** (2008). A versatile strategy to define the phosphorylation preferences of plant protein kinases and screen for putative substrates. Plant Journal 55, 104-117.

**Wang, D.G., Harper, J.F., and Gribskov, M.** (2003). Systematic trans-genomic comparison of protein kinases between *Arabidopsis* and *Saccharomyces cerevisiae*. Plant Physiology 132, 2152-2165.

**Wang, H., Chevalier, D., Larue, C., Ki Cho, S., and Walker, J.C.** (2007). The Protein Phosphatases and Protein Kinases of *Arabidopsis* thaliana. The *Arabidopsis* book / American Society of Plant Biologists 5, e0106-e0106.

**Wang, X., Peng, F., Li, M., Yang, L., and Li, G**. (2012). Expression of a heterologous SnRK1 in tomato increases carbon assimilation, nitrogen uptake and modifies fruit development. Journal of Plant Physiology 169, 1173-1182.

**Warden, S.M., Richardson, C., O'Donnell, J., Stepleton, D., Kemp, B.E., and Witters, L.A.** (2001). Post-translational modifications of the beta-1 subunit of AMP-activated protein kinase affect enzyme activity and cellular localization. Biochemical Journal 354, 275-283.

**Watt, M.J., Steinberg, G.R., Chen, Z.-P., Kemp, B.E., and Febbraio, M.A.** (2006). Fatty acids stimulate AMP-activated protein kinase and enhance fatty acid oxidation in L6 myotubes. Journal of Physiology-London 574, 139-147.

**Williams, S.G., and Hall, K.B.** (2011). Human U2B " protein binding to snRNA stem loops. Biophysical Chemistry 159, 82-89.

**Williams, S.P., Rangarajan, P., Donahue, J.L., Hess, J.E., and Gillaspy, G.E.** (2014). Regulation of Sucrose non-Fermenting Related Kinase 1 genes in *Arabidopsis thaliana*. Frontiers in Plant Science 5.

**Wu, Y., Deng, Z., Lai, J., Zhang, Y., Yang, C., Yin, B., Zhao, Q., Zhang, L., Li, Y., Yang, C., and Xie, Q.** (2009). Dual function of *Arabidopsis* ATAF1 in abiotic and biotic stress responses. Cell Research 19, 1279-1290.

**Xiong, L.M., and Zhu, J.K.** (2001). Abiotic stress signal transduction in plants: Molecular and genetic perspectives. Physiologia Plantarum 112, 152-166.

**Xiong, L.M., Schumaker, K.S., and Zhu, J.K.** (2002). Cell signaling during cold, drought, and salt stress. Plant Cell **14,** S165-S183.

**Yoshida, R., Umezawa, T., Mizoguchi, T., Takahashi, S., Takahashi, F., and Shinozaki, K.** (2006). The regulatory domain of SRK2E/OST1/SnRK2.6 interacts with ABI1 and integrates abscisic acid (ABA) and osmotic stress signals controlling stomatal closure in *Arabidopsis*. Journal of Biological Chemistry 281, 5310-5318.

**Zhang, Y., Primavesi, L.F., Jhurreea, D., Andralojc, P.J., Mitchell, R.A.C., Powers, S.J., Schluepmann, H., Delatte, T., Wingler, A., and Paul, M.J.** (2009). Inhibition of SNF1-Related Protein Kinase1 Activity and Regulation of Metabolic Pathways by Trehalose-6-Phosphate. Plant Physiology 149, 1860-1871.

**Zhang, Y.H., Shewry, P.R., Jones, H., Barcelo, P., Lazzeri, P.A., and Halford, N.G.** (2001). Expression of antisense SnRK1 protein kinase sequence causes abnormal pollen development and male sterility in transgenic barley. Plant Journal 28, 431-441.

**Zhu, J.K.** (2002). Salt and drought stress signal transduction in plants. Annual Review of Plant Biology 53, 247-273.

**Zulawski, M., Braginets, R., and Schulze, W.X.** (2013). PhosPhAt goes kinases-searchable protein kinase target information in the plant phosphorylation site database PhosPhAt. Nucleic Acids Research 41, D1176-D1184.

# Appendix

# Appendix 1

**Table I:** ***Viability of bait, prey library and diploids by plating on selective agar medium.***
*The first dilution (1/10) was made with 50µL of mate solution adding 450µL of sterile water and kanamycin (Kan) (1/100) and the three following serial dilutions was made.*

| Dilution | | N. of colonies in agar medium plate SD–L (pGADT7) | N. of colonies in agar medium plate SD–W (pGBKT7) | N of colonies in medium agar plate SD-L-W (2N cells) |
|---|---|---|---|---|
| ***$10^{-1}$ (1/10)*** | 50µL mate + 450µL $H_2O$ + Kan | uncountable | uncountable | uncountable |
| ***$10^{-2}$ (1/100)*** | 50µL $10^{-1}$ + 450µL $H_2O$ + kan | uncountable | uncountable | 416 |
| ***$10^{-3}$ (1/1,000)*** | 50µL $10^{-2}$ + 450µL $H_2O$ + kan | 828 | uncountable | 38 (35) |
| ***$10^{-4}$ (1/10,000)*** | 50µL $10^{-3}$ + 450µL $H_2O$ +kan | 87 (80) | uncountable | 10 |

**Table II:** ***Positive control of prey Library***
*With 20µL of library and 180µL of sterile water, three more dilutions were made and all plated in SD-L agar plate to count how many library colonies existed in total volume used in mating procedure.*

| Dilution | | N Of Y2h Library Colonies (pGADT7) |
|---|---|---|
| **$10^{-1}$** | 20µL cDNA Library + 180µL $H_2O$ | uncountable |
| **$10^{-2}$** | 20µL $10^{-1}$ + 180µL $H_2O$ | uncountable |
| **$10^{-3}$** | 20µL $10^{-2}$ + 180µL $H_2O$ | 856 |
| **$10^{-4}$** | 20µL $10^{-3}$ + 180µL $H_2O$ | 73 |

**Mating efficiency:**

$$\frac{No\ of\ \frac{cfu}{ml} of\ diploids}{No\ of\ \frac{cfu}{ml} of\ limiting\ partner} \text{ x } 100 = \quad \frac{35*10\text{^}3}{80*10\text{^}4} \text{ x } 100 \approx \mathbf{4.4\%}$$

**Number of diploids**

(1) x (2) x (3) x (4) = 35 x 11.8 x 10 x 1,000= **4,130,000 diploids colonies**

**Where:**

(1) – number of colonies grew on SD-L-W (35)
(2) – resuspension volume (11.8 = total volume of cells + medium) [see "Determination of transformation and mating efficiency" in Materials and Methods]
(3) – plating volume (100µl)
(4) – respective dilution where colonies grew (1/1,000)

# Appendix 2

## Table III: *All the FunCat categories where the 64 proteins identified are inserted.*

*The red color p-Values shows the statistically significance for that matching category.*

| FUNCTIONAL CATEGORY | Abs. SET | Rel. SET (%) | Abs. GENOME | Rel. GENOME (%) | Rel SET/Rel GENOME | P-VALUE |
|---|---|---|---|---|---|---|
| **01 METABOLISM** | 33 | 39,20 | 7084 | 21,20 | 1,85 | **1,24E-04** |
| 01.01 amino acid metabolism | 5 | 5,95 | 1210 | 3,62 | 1,64 | 1,89E-01 |
| 01.01.03 assimilation of ammonia, metabolism of the glutamate group | 2 | 2,38 | 377 | 1,12 | 2,13 | 2,45E-01 |
| 01.01.06 metabolism of the aspartate family | 1 | 1,19 | 181 | 0,54 | 2,20 | 3,67E-01 |
| 01.01.06.05 metabolism of methionine | 1 | 1,19 | 86 | 0,25 | 4,76 | 1,95E-01 |
| 01.01.06.05.01 biosynthesis of methionine | 1 | 1,19 | 61 | 0,18 | 6,61 | 1,43E-01 |
| 01.01.11 metabolism of the pyruvate family (alanine, isoleucine, leucine, valine) and D-alanine | 1 | 1,19 | 109 | 0,32 | 3,72 | 2,40E-01 |
| 01.01.11.02 metabolism of isoleucine | 1 | 1,19 | 42 | 0,12 | 9,92 | 1,00E-01 |
| 01.01.11.02.01 biosynthesis of isoleucine | 1 | 1,19 | 23 | 0,06 | 19,83 | 5,63E-02 |
| 01.01.11.02.02 degradation of isoleucine | 1 | 1,19 | 29 | 0,08 | 14,88 | 7,04E-02 |
| 01.01.11.03 metabolism of valine | 1 | 1,19 | 55 | 0,16 | 7,44 | 1,29E-01 |
| 01.01.11.03.01 biosynthesis of valine | 1 | 1,19 | 22 | 0,06 | 19,83 | 5,39E-02 |
| 01.01.11.03.02 degradation of valine | 1 | 1,19 | 43 | 0,12 | 9,92 | 1,03E-01 |
| 01.01.11.04 metabolism of leucine | 1 | 1,19 | 56 | 0,16 | 7,44 | 1,32E-01 |
| 01.01.11.04.01 biosynthesis of leucine | 1 | 1,19 | 31 | 0,09 | 13,22 | 7,51E-02 |
| 01.01.11.04.02 degradation of leucine | 1 | 1,19 | 40 | 0,11 | 10,82 | 9,59E-02 |
| 01.02 nitrogen, sulfur and selenium metabolism | 4 | 4,76 | 551 | 1,64 | 2,90 | 5,04E-02 |
| 01.02.02 nitrogen metabolism | 1 | 1,19 | 67 | 0,20 | 5,95 | 1,55E-01 |
| 01.02.03 sulfur metabolism | 1 | 1,19 | 42 | 0,12 | 9,92 | 1,00E-01 |
| 01.02.03.01 sulfate assimilation | 1 | 1,19 | 23 | 0,06 | 19,83 | 5,63E-02 |
| 01.03 nucleotide/nucleoside/nucleobase metabolism | 3 | 3,57 | 1013 | 3,03 | 1,18 | 4,71E-01 |
| 01.03.01 purin nucleotide/nucleoside/nucleobase metabolism | 2 | 2,38 | 308 | 0,92 | 2,59 | 1,82E-01 |
| 01.04 phosphate metabolism | 10 | 11,90 | 1406 | 4,20 | 2,83 | **2,76E-03** |
| 01.05 C-compound and carbohydrate metabolism | 17 | 20,20 | 2744 | 8,21 | 2,46 | **4,20E-04** |
| 01.05.02 sugar, glucoside, polyol and carboxylate metabolism | 9 | 10,70 | 640 | 1,91 | 5,60 | **3,38E-05** |
| 01.05.02.04 sugar, glucoside, polyol and carboxylate anabolism | 1 | 1,19 | 118 | 0,35 | 3,40 | 2,57E-01 |
| 01.05.02.07 sugar, glucoside, polyol and carboxylate catabolism | 7 | 8,33 | 350 | 1,04 | 8,01 | **2,96E-05** |
| 01.05.03 polysaccharide metabolism | 1 | 1,19 | 280 | 0,83 | 1,43 | 5,07E-01 |
| 01.05.06 C-2 compound and organic acid metabolism | 1 | 1,19 | 71 | 0,21 | 5,67 | 1,64E-01 |
| 01.05.06.07 C-2 compound and organic acid catabolism | 1 | 1,19 | 53 | 0,15 | 7,93 | 1,25E-01 |
| 01.05.25 regulation of C-compound and carbohydrate metabolism | 3 | 3,57 | 219 | 0,65 | 5,49 | **1,80E-02** |
| 01.06 lipid, fatty acid and isoprenoid metabolism | 6 | 7,14 | 1608 | 4,81 | 1,48 | 2,18E-01 |
| 01.06.05 fatty acid metabolism | 6 | 7,14 | 206 | 0,61 | 11,70 | **1,39E-05** |
| 01.07 metabolism of vitamins, cofactors, and prosthetic groups | 4 | 4,76 | 536 | 1,60 | 2,98 | **4,64E-02** |
| 01.07.01 biosynthesis of vitamins, cofactors, and prosthetic groups | 3 | 3,57 | 318 | 0,95 | 3,76 | **4,63E-02** |
| 01.20 secondary metabolism | 2 | 2,38 | 1445 | 4,32 | 0,55 | 8,83E-01 |
| 01.20.17 metabolism of secondary products derived from primary amino acids | 1 | 1,19 | 275 | 0,82 | 1,45 | 5,01E-01 |
| 01.20.17.07 metabolism of glycosinolates and derivatives | 1 | 1,19 | 158 | 0,47 | 2,53 | 3,29E-01 |
| 01.20.19 metabolism of secondary products derived from glycine, L-serine and L-alanine | 1 | 1,19 | 51 | 0,15 | 7,93 | 1,21E-01 |
| 01.20.19.01 metabolism of porphyrins | 1 | 1,19 | 39 | 0,11 | 10,82 | 9,36E-02 |
| 01.20.35 metabolism of secondary products derived from L-phenylalanine and L-tyrosine | 1 | 1,19 | 376 | 1,12 | 1,06 | 6,14E-01 |
| 01.20.35.01 metabolism of phenylpropanoids | 1 | 1,19 | 374 | 1,11 | 1,07 | 6,12E-01 |

## *Table III: (continue)*

| | | | | | | |
|---|---|---|---|---|---|---|
| **02 ENERGY** | 14 | 16,60 | 1484 | 4,44 | 3,74 | **1,89E-05** |
| 02.01 glycolysis and gluconeogenesis | 4 | 4,76 | 295 | 0,88 | 5,41 | **6,62E-03** |
| 02.07 pentose-phosphate pathway | 4 | 4,76 | 98 | 0,29 | 16,41 | **1,12E-04** |
| 02.10 tricarboxylic-acid pathway (citrate cycle, Krebs cycle, TCA cycle) | 2 | 2,38 | 116 | 0,34 | 7,00 | **3,47E-02** |
| 02.11 electron transport and membrane-associated energy conservation | 1 | 1,19 | 336 | 1,00 | 1,19 | 5,73E-01 |
| 02.13 respiration | 1 | 1,19 | 299 | 0,89 | 1,34 | 5,31E-01 |
| 02.16 fermentation | 1 | 1,19 | 63 | 0,18 | 6,61 | 1,47E-01 |
| 02.25 oxidation of fatty acids | 6 | 7,14 | 73 | 0,21 | 34,00 | **3,14E-08** |
| **10 CELL CYCLE AND DNA PROCESSING** | 4 | 4,76 | 2520 | 7,54 | 0,63 | 8,86E-01 |
| 10.01 DNA processing | 2 | 2,38 | 1541 | 4,61 | 0,52 | 9,05E-01 |
| 10.01.05 DNA recombination and DNA repair | 1 | 1,19 | 670 | 2,00 | 0,60 | 8,18E-01 |
| 10.01.05.01 DNA repair | 1 | 1,19 | 541 | 1,61 | 0,74 | 7,47E-01 |
| 10.01.09 DNA restriction or modification | 1 | 1,19 | 883 | 2,64 | 0,45 | 8,95E-01 |
| 10.01.09.05 DNA conformation modification (e.g. chromatin) | 1 | 1,19 | 845 | 2,52 | 0,47 | 8,84E-01 |
| 10.03 cell cycle | 2 | 2,38 | 1495 | 4,47 | 0,53 | 8,95E-01 |
| 10.03.01 mitotic cell cycle and cell cycle control | 1 | 1,19 | 1086 | 3,25 | 0,37 | 9,38E-01 |
| 10.03.02 meiosis | 1 | 1,19 | 251 | 0,75 | 1,59 | 4,70E-01 |
| **11 TRANSCRIPTION** | 8 | 9,52 | 5043 | 15,00 | 0,63 | 9,51E-01 |
| 11.02 RNA synthesis | 7 | 8,33 | 4510 | 13,50 | 0,62 | 9,47E-01 |
| 11.02.01 rRNA synthesis | 1 | 1,19 | 145 | 0,43 | 2,77 | 3,06E-01 |
| 11.02.02 tRNA synthesis | 1 | 1,19 | 135 | 0,40 | 2,98 | 2,89E-01 |
| 11.02.03 mRNA synthesis | 7 | 8,33 | 4354 | 13,00 | 0,64 | 9,33E-01 |
| 11.02.03.01 general transcription activities | 1 | 1,19 | 338 | 1,01 | 1,18 | 5,75E-01 |
| 11.02.03.04 transcriptional control | 6 | 7,14 | 3616 | 10,80 | 0,66 | 9,03E-01 |
| 11.04 RNA processing | 1 | 1,19 | 1147 | 3,43 | 0,35 | 9,47E-01 |
| 11.04.03 mRNA processing (splicing, 5^-, 3^-end processing) | 1 | 1,19 | 796 | 2,38 | 0,50 | 8,68E-01 |
| 11.04.03.01 splicing | 1 | 1,19 | 632 | 1,89 | 0,63 | 7,99E-01 |
| **12 PROTEIN SYNTHESIS** | 2 | 2,38 | 1311 | 3,92 | 0,61 | 8,47E-01 |
| 12.01 ribosome biogenesis | 2 | 2,38 | 664 | 1,98 | 1,20 | 5,00E-01 |
| 12.01.01 ribosomal proteins | 2 | 2,38 | 502 | 1,50 | 1,59 | 3,61E-01 |
| 12.04 translation | 1 | 1,19 | 713 | 2,13 | 0,56 | 8,37E-01 |
| **14 PROTEIN FATE (folding, modification, destination)** | 13 | 15,40 | 4384 | 13,10 | 1,18 | 3,06E-01 |
| 14.01 protein folding and stabilization | 3 | 3,57 | 720 | 2,15 | 1,66 | 2,72E-01 |
| 14.04 protein targeting, sorting and translocation | 3 | 3,57 | 865 | 2,58 | 1,38 | 3,71E-01 |
| 14.07 protein modification | 7 | 8,33 | 2341 | 7,00 | 1,19 | 3,75E-01 |
| 14.07.03 modification by phosphorylation, dephosphorylation, autophosphorylation | 5 | 5,95 | 683 | 2,04 | 2,92 | **2,90E-02** |
| 14.07.05 modification by ubiquitination, deubiquitination | 2 | 2,38 | 587 | 1,75 | 1,36 | 4,36E-01 |
| 14.10 assembly of protein complexes | 1 | 1,19 | 586 | 1,75 | 0,68 | 7,74E-01 |
| 14.13 protein/peptide degradation | 3 | 3,57 | 1316 | 3,94 | 0,91 | 6,48E-01 |
| 14.13.01 cytoplasmic and nuclear protein degradation | 1 | 1,19 | 701 | 2,09 | 0,57 | 8,32E-01 |
| 14.13.01.01 proteasomal degradation (ubiquitin/proteasomal pathway) | 1 | 1,19 | 545 | 1,63 | 0,73 | 7,49E-01 |
| 14.13.04 lysosomal and vacuolar protein degradation | 1 | 1,19 | 101 | 0,30 | 3,97 | 2,25E-01 |

## *Table III: (continue)*

| | | | | | | |
|---|---|---|---|---|---|---|
| **16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)** | 27 | 32,10 | 7436 | 22,20 | 1,45 | **2,35E-02** |
| 16.01 protein binding | 17 | 20,20 | 4712 | 14,10 | 1,43 | 7,70E-02 |
| 16.03 nucleic acid binding | 6 | 7,14 | 1991 | 5,96 | 1,20 | 3,85E-01 |
| 16.03.01 DNA binding | 4 | 4,76 | 1027 | 3,07 | 1,55 | 2,59E-01 |
| 16.03.03 RNA binding | 2 | 2,38 | 995 | 2,97 | 0,80 | 7,18E-01 |
| 16.09 lipid binding | 1 | 1,19 | 177 | 0,52 | 2,29 | 3,60E-01 |
| 16.17 metal binding | 6 | 7,14 | 2039 | 6,10 | 1,17 | 4,07E-01 |
| 16.17.01 calcium binding | 1 | 1,19 | 315 | 0,94 | 1,27 | 5,49E-01 |
| 16.17.09 heavy metal binding (Cu, Fe, Zn) | 5 | 5,95 | 1286 | 3,85 | 1,55 | 2,23E-01 |
| 16.19 nucleotide/nucleoside/nucleobase binding | 9 | 10,70 | 2245 | 6,72 | 1,59 | 1,11E-01 |
| 16.19.03 ATP binding | 6 | 7,14 | 1589 | 4,75 | 1,50 | 2,10E-01 |
| 16.19.05 GTP binding | 2 | 2,38 | 344 | 1,02 | 2,33 | 2,14E-01 |
| 16.21 complex cofactor/cosubstrate/vitamine binding | 3 | 3,57 | 772 | 2,31 | 1,55 | 3,07E-01 |
| 16.21.05 FAD/FMN binding | 1 | 1,19 | 180 | 0,53 | 2,25 | 3,65E-01 |
| 16.21.07 NAD/NADP binding | 1 | 1,19 | 292 | 0,87 | 1,37 | 5,22E-01 |
| 16.21.17 pyridoxal phosphate binding | 1 | 1,19 | 115 | 0,34 | 3,50 | 2,52E-01 |
| **18 REGULATION OF METABOLISM AND PROTEIN FUNCTION** | 6 | 7,14 | 1407 | 4,21 | 1,70 | 1,43E-01 |
| 18.01 regulation by | 5 | 5,95 | 833 | 2,49 | 2,39 | 5,90E-02 |
| 18.01.01 regulation by modification | 5 | 5,95 | 434 | 1,29 | 4,61 | **4,82E-03** |
| 18.02 regulation of protein activity | 6 | 7,14 | 1380 | 4,13 | 1,73 | 1,34E-01 |
| 18.02.01 enzymatic activity regulation / enzyme regulator | 1 | 1,19 | 860 | 2,57 | 0,46 | 8,89E-01 |
| 18.02.01.01 enzyme activator | 1 | 1,19 | 339 | 1,01 | 1,18 | 5,76E-01 |
| 18.02.09 regulator of transcription factor | 3 | 3,57 | 312 | 0,93 | 3,84 | **4,42E-02** |
| **20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES** | 11 | 13,00 | 4048 | 12,10 | 1,07 | 4,41E-01 |
| 20.01 transported compounds (substrates) | 8 | 9,52 | 2514 | 7,52 | 1,27 | 2,97E-01 |
| 20.01.01 ion transport | 3 | 3,57 | 664 | 1,98 | 1,80 | 2,34E-01 |
| 20.01.01.01 cation transport (H+, Na+, K+, Ca2+ , NH4+, etc.) | 1 | 1,19 | 478 | 1,43 | 0,83 | 7,02E-01 |
| 20.01.01.07 anion transport | 2 | 2,38 | 201 | 0,60 | 3,97 | 9,12E-02 |
| 20.01.01.07.07 phosphate transport | 1 | 1,19 | 66 | 0,19 | 6,26 | 1,53E-01 |
| 20.01.10 protein transport | 4 | 4,76 | 618 | 1,85 | 2,57 | 7,06E-02 |
| 20.01.15 electron transport | 1 | 1,19 | 570 | 1,70 | 0,70 | 7,65E-01 |
| 20.03 transport facilities | 5 | 5,95 | 1493 | 4,46 | 1,33 | 3,22E-01 |
| 20.03.01 channel / pore class transport | 2 | 2,38 | 190 | 0,56 | 4,25 | 8,29E-02 |
| 20.03.01.01 ion channels | 2 | 2,38 | 130 | 0,38 | 6,26 | **4,26E-02** |
| 20.03.25 ABC transporters | 1 | 1,19 | 178 | 0,53 | 2,25 | 3,62E-01 |
| 20.09 transport routes | 6 | 7,14 | 2224 | 6,65 | 1,07 | 4,91E-01 |
| 20.09.04 mitochondrial transport | 1 | 1,19 | 219 | 0,65 | 1,83 | 4,25E-01 |
| 20.09.07 vesicular transport (Golgi network, etc.) | 3 | 3,57 | 750 | 2,24 | 1,59 | 2,92E-01 |
| 20.09.07.27 vesicle fusion | 2 | 2,38 | 94 | 0,28 | 8,50 | **2,35E-02** |
| 20.09.13 vacuolar/lysosomal transport | 3 | 3,57 | 423 | 1,26 | 2,83 | 9,10E-02 |
| 20.09.16 cellular export and secretion | 3 | 3,57 | 318 | 0,95 | 3,76 | **4,63E-02** |
| 20.09.16.09 vesicular cellular export | 2 | 2,38 | 84 | 0,25 | 9,52 | **1,91E-02** |
| 20.09.18 cellular import | 3 | 3,57 | 435 | 1,30 | 2,75 | 9,69E-02 |
| 20.09.18.09 vesicular cellular import | 3 | 3,57 | 244 | 0,73 | 4,89 | **2,38E-02** |
| 20.09.18.09.01 endocytosis | 3 | 3,57 | 244 | 0,73 | 4,89 | **2,38E-02** |
| **30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM** | 9 | 10,70 | 3205 | 9,59 | 1,12 | 4,16E-01 |
| 30.01 cellular signalling | 9 | 10,70 | 3023 | 9,05 | 1,18 | 3,49E-01 |
| 30.01.05 enzyme mediated signal transduction | 6 | 7,14 | 821 | 2,45 | 2,91 | **1,74E-02** |
| 30.01.05.05 G-protein mediated signal transduction | 1 | 1,19 | 366 | 1,09 | 1,09 | 6,04E-01 |
| 30.01.05.05.01 small GTPase mediated signal transduction | 1 | 1,19 | 255 | 0,76 | 1,57 | 4,75E-01 |
| 30.01.09 second messenger mediated signal transduction | 4 | 4,76 | 305 | 0,91 | 5,23 | **7,42E-03** |
| 30.01.09.03 Ca2+ mediated signal transduction | 1 | 1,19 | 94 | 0,28 | 4,25 | 2,11E-01 |
| 30.01.09.07 cAMP/cGMP mediated signal transduction | 3 | 3,57 | 14 | 0,04 | 89,25 | **5,47E-06** |

## *Table III: (continue)*

| | | | | | | |
|---|---|---|---|---|---|---|
| **32 CELL RESCUE, DEFENSE AND VIRULENCE** | 13 | 15,40 | 3685 | 11,00 | 1,40 | 1,31E-01 |
| 32.01 stress response | 13 | 15,40 | 2108 | 6,31 | 2,44 | **2,27E-03** |
| 32.01.01 oxidative stress response | 2 | 2,38 | 226 | 0,67 | 3,55 | 1,11E-01 |
| 32.01.03 osmotic and salt stress response | 1 | 1,19 | 68 | 0,20 | 5,95 | 1,58E-01 |
| 32.01.05 heat shock response | 1 | 1,19 | 112 | 0,33 | 3,61 | 2,46E-01 |
| 32.01.07 unfolded protein response (e.g. ER quality control) | 2 | 2,38 | 150 | 0,44 | 5,41 | 5,50E-02 |
| 32.01.11 nutrient starvation response | 4 | 4,76 | 42 | 0,12 | 39,67 | **3,87E-06** |
| 32.07 detoxification | 2 | 2,38 | 720 | 2,15 | 1,11 | 5,43E-01 |
| 32.07.03 detoxification by modification | 2 | 2,38 | 116 | 0,34 | 7,00 | **3,47E-02** |
| 32.07.07 oxygen and radical detoxification | 2 | 2,38 | 165 | 0,49 | 4,86 | 6,50E-02 |
| 32.07.07.07 superoxide metabolism | 2 | 2,38 | 30 | 0,08 | 29,75 | **2,60E-03** |
| **34 INTERACTION WITH THE ENVIRONMENT** | 6 | 7,14 | 1325 | 3,96 | 1,80 | 1,17E-01 |
| 34.01 homeostasis | 2 | 2,38 | 528 | 1,58 | 1,51 | 3,84E-01 |
| 34.01.01 homeostasis of cations | 1 | 1,19 | 370 | 1,10 | 1,08 | 6,08E-01 |
| 34.01.01.01 homeostasis of metal ions (Na, K, Ca etc.) | 1 | 1,19 | 238 | 0,71 | 1,68 | 4,52E-01 |
| 34.01.03 homeostasis of anions | 1 | 1,19 | 58 | 0,17 | 7,00 | 1,36E-01 |
| 34.07 cell adhesion | 3 | 3,57 | 72 | 0,21 | 17,00 | **8,07E-04** |
| 34.07.02 cell-matrix adhesion | 3 | 3,57 | 8 | 0,02 | 178,50 | **8,51E-07** |
| 34.11 cellular sensing and response to external stimulus | 1 | 1,19 | 506 | 1,51 | 0,79 | 7,23E-01 |
| 34.11.09 temperature perception and response | 1 | 1,19 | 69 | 0,20 | 5,95 | 1,60E-01 |
| **40 CELL FATE** | 6 | 7,14 | 1350 | 4,04 | 1,77 | 1,24E-01 |
| 40.01 cell growth / morphogenesis | 3 | 3,57 | 827 | 2,47 | 1,45 | 3,45E-01 |
| 40.01.05 growth regulators / regulation of cell size | 3 | 3,57 | 185 | 0,55 | 6,49 | **1,15E-02** |
| 40.10 cell death | 1 | 1,19 | 491 | 1,47 | 0,81 | 7,12E-01 |
| 40.10.02 apoptosis (type I programmed cell death) | 1 | 1,19 | 422 | 1,26 | 0,94 | 6,57E-01 |
| 40.10.02.01 anti-apoptosis | 1 | 1,19 | 85 | 0,25 | 4,76 | 1,93E-01 |
| 40.20 cell aging | 5 | 5,95 | 117 | 0,35 | 17,00 | **1,20E-05** |
| **41 DEVELOPMENT (Systemic)** | 1 | 1,19 | 621 | 1,85 | 0,64 | 7,94E-01 |
| 41.03 plant development | 1 | 1,19 | 621 | 1,85 | 0,64 | 7,94E-01 |
| **42 BIOGENESIS OF CELLULAR COMPONENTS** | 8 | 9,52 | 3413 | 10,20 | 0,93 | 6,36E-01 |
| 42.01 cell wall | 4 | 4,76 | 824 | 2,46 | 1,93 | 1,54E-01 |
| 42.04 cytoskeleton/structural proteins | 1 | 1,19 | 525 | 1,57 | 0,76 | 7,36E-01 |
| 42.10 nucleus | 1 | 1,19 | 996 | 2,98 | 0,40 | 9,22E-01 |
| 42.10.03 organization of chromosome structure | 1 | 1,19 | 892 | 2,67 | 0,45 | 8,97E-01 |
| 42.25 vacuole or lysosome | 2 | 2,38 | 144 | 0,43 | 5,53 | 5,11E-02 |
| **43 CELL TYPE DIFFERENTIATION** | 6 | 7,14 | 584 | 1,74 | 4,10 | **3,58E-03** |
| 43.01 fungal/microorganismic cell type differentiation | 6 | 7,14 | 584 | 1,74 | 4,10 | **3,58E-03** |
| 43.01.03 fungal and other eukaryotic cell type differentiation | 6 | 7,14 | 584 | 1,74 | 4,10 | **3,58E-03** |
| 43.01.03.05 budding, cell polarity and filament formation | 5 | 5,95 | 288 | 0,86 | 6,92 | **8,15E-04** |
| 43.01.03.09 development of asco- basidio- or zygospore | 1 | 1,19 | 295 | 0,88 | 1,35 | 5,26E-01 |
| **99 UNCLASSIFIED PROTEINS** | 29 | 34,50 | 14259 | 42,60 | 0,81 | 9,49E-01 |

# Appendix 3

MAMQSLSPVPKLLSTTPSSVLSSDKNFFFVDFVGLYCKSKRTRRRLRGDSSSSSRSSSSLSRLSSVRAVIDLERVHGVSEKDLSSPSALRPQVRFFTDINFTNTQRAKFHPLWGSFKQVANLEDILSERGACGV
GFIANLDNIPSHGVVKDALIALGCMEHRGGCGADNDSGDGSGLMSSIPWDFFNVWAKEQSLAPFDKLHTGVGMIFLPQDDTFMQEAKQVIENIFEKEGLQVLGWREVPVNVPIVGKNARETMPNIQQ
VFVKIAKEDSTDDIERELYICRKLIERAVATESWGTELYFCSLSNQTIVYKGMLRSEALGLFYLDLQNELYESPFAIYHRRYSTNTSPRWPLAQPMRFLGHNGEINTIQGNLNWMQSREASLKAAVWNGREN
EIRPFGNPRGSDSANLDSAAEIMIRSGRTPEEALMILVPEAYKNHPTLSVKYPEVVDFYDYYKGQMEAWDGPALLLFSDGKTVGACLDRNGLRPARYWRTSDNFVYVASEVGVVPVDEAKVTMKGRLGP
GMMIAVDLVNGQVYENTEVKKRISSFNPYGKWIKENSRFLKPVNFKSSTVMENEEILRSQQAFGYSSEDVQMVIESMASQGKEPTFCMGDDIPLAGLSQRPHMLYDYFKQRFAQVTNPAIDPLREGLVM
SLEVNIGKRGNILELGPENASQVILSNPVLNEGALEELMKDQYLKPKVLSTYFDIRKGVEGSLQKALYYLCEAADDAVRSGSQLLVLSDRSDRLEPTRPSIPIMLAVGAVHQHLIQNGLRMSASIVADTAQCFS
THHFACLVGYGASAVCPYLALETCRQWRLSNKTVAFMRNGKIPTVTIEQAQKNYTKAVNAGLLKILSKMGISLLSSYCGAQIFEIYGLGQDVVDLAFTGSVSKISGLTFDELARETLSFWVKAFSEDTTKRLEN
FGFIQFRPGGEYHSNNPEMSKLLHKAVREKSETAYAVYQQHLSNRPVNVLRDLLEFKSDRAPIPVGKVEPAVAIVQRFCTGGMSLGAISRETHEAIAIAMNRIGGKSNSGEGGEDPIRWKPLTDVVDGYSP
TLPHLKGLQNGDIATSAIKQVASGRFGVTPTFLVNADQLEIKVAQGAKPGEGGQLPGKKVSAYIARLRSSKPGVPLISPPPHHDIYSIEDLAQLIFDLHQINPNAKVSVKLVAEAGIGTVASGVAKGNADIIQIS
GHDGGTGASPISSIKHAGGPWELGLTETHQTLIANGLRERVILRVDGGLKSGVDVLMAAAAMGADEYGFGSLAMIATGCVMARICHTNNCPVGVASQREELRARFPGVPGDLVNYFLYVAEEVRGILAQL
GYNSLDDIIGRTELLRPRDISLVKTQHLDLSYLLSSVGTPSLSSTEIRKQEVHTNGPVLDDDILADPLVIDAIENEKVVEKTVKICNVDRAACGRVAGVIAKKYGDTGFAGQVNLTFLGSAGQSFGCFLIPGMNI
RLIGESNDYVGKGMAGGEIVVTPVEKIGFVPEEATIVGNTCLYGATGGQIFARGKAGERFAVRNSLAEAVVEGTGDHCCEYMTGGCVVVLGKVGRNVAAGMTGGLAYLLDEDDTLLPKINREIVKIQRVT
APAGELQLKSLIEAHVEKTGSSKGATILNEWEKYLPLFWQLVPPSEEDTPEASAAYVRTSTGEVTFQSA

***Figure 1: Protein sequence of GLU1 (AT5G04140.1)***

*GLU1 protein sequence is composed by 1622 aa.*

*Highlighted yellow text specify the S/T residues that has been detected in the phosphorylated form (experiment data for AT5G04140.1 – PhosPhAt 4.0 database). The blue letters indicate the putative SnRK1 recognition sequence ([hydrophobic]X[R/K]XX[S/T]XXX[hydrophobic]) (Vlad et al., 2008).*