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CROSSTALK BETWEEN ENERGY AND GIBBERELLIN SIGNALING PATHWAYS – A MOLECULAR APPROACH

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Marco Aurélio Dias Simões

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Obrigado!

RESUMO

Resumo

As plantas, como todos os organismos, têm de enfrentar uma grande variedade de stresses bióticos e abióticos, mas sendo organismos sésseis elas não são capazes de escapar a estas condições adversas. Portanto, a sua sobrevivência é criticamente depende da sua resposta eficiente às mudanças ambientais. Direta ou indiretamente, o stress geralmente leva a um défice de energia. Se a homeostasia energética não for restabelecida, o crescimento e o desenvolvimento da planta são drasticamente comprometidos. SNF1 (sucrose non-fermenting 1) -related protein kinase 1.1 (SnRK1.1) desempenha um papel importante na coordenação das respostas de energia e stress. Esta kinase deteta e sinaliza condições de stress, tais como escuridão inesperada, baixo teor de açúcar e outros stresses, atuando em genes que controlam uma rede de genes de transcrição. Nas últimas décadas, vários estudos têm revelado um cruzamento entre as vias de sinalização do açúcar e das hormonas. Um estudo recente identificou as proteínas DELLA como um interator de SnRK1 na presença de NDF4, utilizando o sistema Y2H. As proteínas DELLA são reguladoras negativas da via de sinalização da giberelina. As giberelinas são hormonas vegetais que controlam vários processos de desenvolvimento, como a germinação e o alongamento do caule. Neste trabalho, nós confirmámos *in vivo* e *in vitro* a interação entre SnRK1.1 e as proteínas DELLA e demonstrámos que esta interação é independente da presença de NDF4. As análises fenotípicas de mutantes sobre-expressores de SnRK1.1 revelaram que este mutante é insensível a giberelinas e hipersensível a paclobrutazol. Estes resultados podem sugerir que SnRK1 interage e fosforila as proteínas DELLA e que esta fosforilação aumenta a sua estabilidade e consequentemente diminui a resposta a giberelinas.

Palavras-chave: SnRK1.1, giberelinas, DELLA, NDF4, fosforilação

ABSTRACT

Abstract

Plants, like all organisms, have to face a wide variety of biotic and abiotic stresses, but being sessile organisms they are not able to escape from these adverse conditions. Therefore, their survival is critically dependent on an efficient response to the environmental changes. Directly or indirectly, stress generally leads to an energy deficit. If energy homeostasis is not reestablished plant growth and development are dramatically compromised. SNF1 (sucrose non-fermenting 1)-related protein kinase 1.1 (SnRK1.1) plays a key role in the coordination of the energy and stress responses. This kinase detects and signals energy stress conditions like unexpected darkness, low sugar and other stresses, acting in genes that control a network of genes transcription. In the last decades, several studies have revealed a crosstalk between sugar and hormones signaling pathways. A recent study identified the DELLA proteins as an interactor of SnRK1 in the presence of NDF4, using the Y2H system. The DELLA protein are negative regulators of the gibberellin signaling pathway. Gibberellins are plant hormones that control several developmental processes like germination and stem elongation. In this work we confirmed *in vitro* and *in vivo* the interaction between SnRK1.1 and the DELLA proteins and we show that this interaction is independent of presence of NDF4. The phenotypic analysis of SnRK1.1 overexpression mutants revealed that this mutant is insensitive to gibberellin and hypersensitive to paclobutrazol. These results might suggest that SnRK1 interacts and phosphorylate the DELLA proteins and that this phosphorylation increases their stability and consequently impairs the response to gibberellin.

Key words: SnRK1.1, gibberellins, DELLA, NDF4, phosphorylation

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Abbreviations and acronyms list

PCR- Polymerase chain reaction

cDNA- Complementary DNA

µL- Microliter

µg- Microgram

aa- Amino acid

mM- Millimolar

PEG- Polyethylene glycol

SD- Yeast minimal media for culturing *Saccharomyces cerevisiae*

L or Leu- Leucine

W or Trp- Tryptophan

A- Adenine

H or HIS- Histidine

AT- Aminotriazole

MS- Murashige-Skoog

GA_3- Gibberellins

ABA- Abscisic acid

PAC- Paclobrutazol

WT- Wild type

SnRK1 OX- SnRK1.1 overexpressor

Ser- Serine

Thr- Threonine

Tyr- Tyrosine

INTRODUCTION

1. Introduction

Energetic and metabolic homeostasis control is a challenge for all living organisms. There is a close relationship between the energy available to organisms and their stress tolerance, survival, cellular growth and longevity (Kenyon, 2005; Baena-Gonzalez et al., 2007). Plants are exposed to different types of abiotic and biotic stresses (Fujita et al., 2006). Associated to stress there is frequently a reduction in photosynthesis and/or respiration, causing energy deficit which leads to physiological, metabolic and molecular responses (Smith and Stitt, 2007). In the last decade, increasing data suggests that SNF1 (sucrose non-fermenting 1)-related protein kinases1 (SnRKs1) interact with nutrient and metabolic signaling pathways, contributing to coordinate the energy and stress responses (Radchuk et al., 2006; Baena-Gonzalez et al., 2007; Polge and Thomas, 2007; Baena-Gonzalez and Sheen, 2008).

SnRK is a large family of protein kinases involved in the response to stress signals in plants that is divided in three sub-families, SnRK1, SnKR2 and SnRK3 (Fig. 1.1) (Hrabak et al., 2003). The SnRK1 subfamily comprises SnRK1.1 (KIN10/AKIN10), SnRK1.2 (KIN11/AKIN11) and SnRK1.3 (KIN12/AKIN12) (Baena-Gonzalez et al., 2007). The closest orthologs of these three proteins are the sucrose-nonfermenting1 (SNF1) in yeast, and AMP-activated protein kinase (AMPK) in mammals. The SnRK1 subfamily is involved in the detection and signaling of apparently unrelated conditions like unexpected darkness conditions, low sugar and stress, acting in a number of genes that control a network of genes transcription (Halford et al., 2003; Baena-Gonzalez et al., 2007). SnRK2 and SnRK3 subfamilies comprise 35 proteins unique to plants, which are involved in responses to salt, osmotic, sugar, drought and cold stresses and abcisic acid (ABA) signaling (Guo et al., 2001; Gong et al., 2002b; Gong et al., 2002a; Guo et al., 2002). It has been suggested that divergence of these two subfamilies from AMPK and SNF1 is a result of gene duplication and a rapid evolution, taking on new roles to enable plants to link metabolic and stress signaling (Coello et al., 2011).

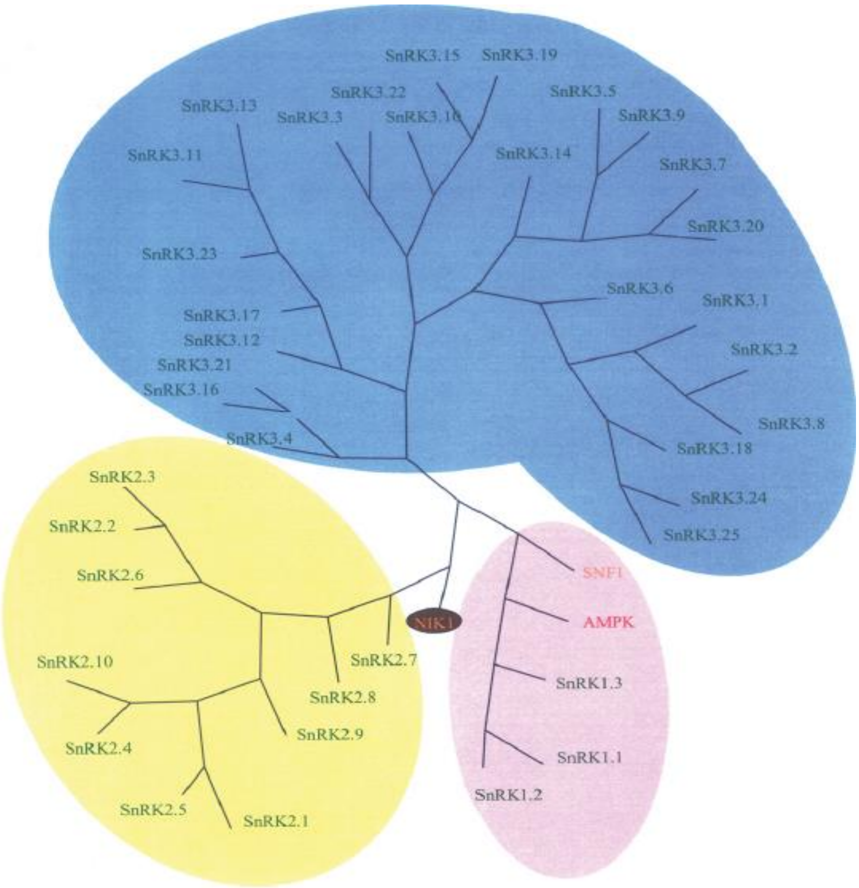


Figure 1.1- SnRK superfamily members of *Arabidopsis thaliana*.
In *A. thalinana* there are three SnRK sub-families, SnRK1, SnKR2 and SnRK3. SnRK1s have two orthologs, AMPK from humans (*Homo sapiens*) and SNF1 from budding yeast (*Saccharomyces cerevisiae*) (Halford and Hey, 2009).

SNF1/AMPK/SnRK1 are metabolic sensors conserved in all eukaryotes (Crozet et al., 2014). Their primary role consist in the evaluation of nutrient availability, environmental stress signaling and energy consumption, in order to induce the required adaptations to maintain energy homeostasis and cell survival (Ghillebert et al., 2011). They regulate the energy balance inactivating anabolic pathways (ATP-consumption) and activating catabolic pathways (ATP-production) through a dual control over cellular metabolism, gene expression regulation and direct phosphorylation of key enzymes (Polge and Thomas, 2007).

SNF1/AMPK/SnRK1 have in common a heterotrimeric structure composed by three subunits, α, β and γ (Fig. 1.2). Various studies demonstrated that the α-subunit activity is dependent of the presence of all other subunits (Carlson et al., 1981; Dyck et al., 1996; Woods et al., 1996; Schmidt and McCartney, 2000; Polge et al., 2008). The catalytic α-subunit displays the highest degree of cross-species conservation, especially in the kinase domain located in the N-terminal half of the protein (Carling et al., 1994; Halford et al., 2003). This subunit is composed by 11 different subdomains, one of which contains the activation loop, or T-loop, essential for the kinase activity (Hanks and Hunter, 1995). The β- and γ- subunits have a regulatory function. The γ-subunit is responsible for the control of the α-subunit activity, while β-subunit serve as scaffold to keep the two subunits together (Crozet et al., 2014). In mammals, γ-subunits have two adenylate binding domains (Hedbacker and Carlson, 2008; Steinberg and Kemp, 2009). Mammalian AMPK is regulated by AMP, which promotes the phosphorylation of the catalytic a-subunit by upstream kinases and protect against dephosphorylation (Davies et al., 1995; Suter et al., 2006; Sanders et al., 2007). SNF1 and SnRK1 are not allosterically activated by AMP (Mitchelhill et al., 1994; Oakhill et al., 2010) and the specific signals that trigger their activation is still unknown. However it has been proposed that, AMP blocks SnRK1 inactivation by preventing dephosphorylation of the T-loop (Sugden et al., 1999a). In yeast and mammals, catalytic subunits contain an auto-inhibitory sequence (AIS) that was shown to inhibit the kinase activity (Hedbacker and Carlson, 2008; Chen et al., 2009). In plants this region apparently doesn’t present an inhibitory function, but contains an ubiquitin associated domain (UBA) that might mediate the interaction with ubiquitinated proteins (Farras et al., 2001). These three protein kinases also present a kinase-associated1 domain (KA1), responsible for the interaction with the regulatory subunits and the upstream phosphatases (Kleinow et al., 2000; Amodeo et al., 2007).

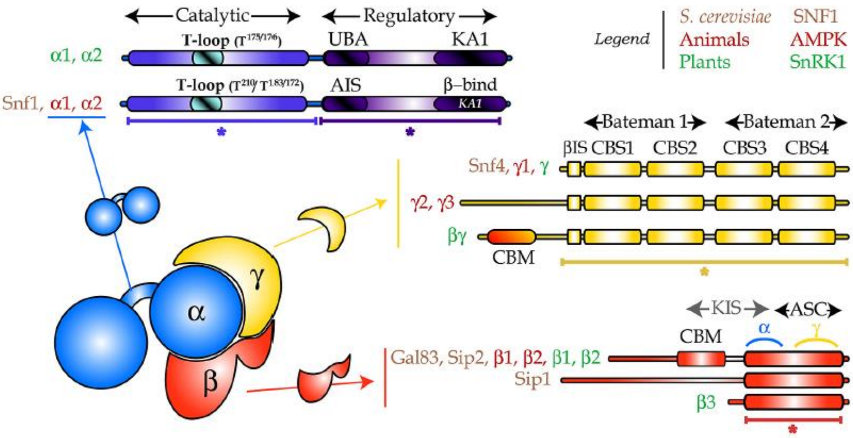


Figure 1.2– Constitutive subunits of SnRK1 proteins of *Arabidopsis thaliana* and their orthologs, AMPK in *Homo sapiens* and SNF1 in *Saccharomyces cerevisiae*.

The α-subunit is represented in blue, β-subunit is represented in red and γ-subunit is represented in yellow (Crozet et al., 2014).

As discussed above, SNF1/AMPK/SnRK1 are metabolic sensors responsible for maintaining the energy homeostasis of organisms.

SNF1 was identified in *S. cerevisiae*, when the *snf1* mutation was recovered from a screening for mutants unable to utilize sucrose (Carlson et al., 1981). This mutant cannot grow without glucose, even in the presence of alternative carbon sources, such as sucrose, galactose, maltose, or non-fermentable carbon sources, like glycerol. This mutant is also impaired in other aspects of cell growth, as glycogen synthesis and sensitivity to heat stress (Carlson et al., 1981; Woods et al., 1994). In glucose limitation situation wild type SNF1 is activated and has a key role in the diauxic shift from fermentative metabolism to oxidative metabolism, controlling the expression of genes necessary to the use of alternative carbon sources less preferred than glucose to produce ATP (DeRisi et al., 1997; Ruiz et al., 2012). Besides its role in the response to glucose limitation, SNF1 is also important in cellular developmental processes (meiosis and sporulation, aging, haploid invasive growth and diploid pseudohyphal growth) and in the response to environmental stresses (salt stress, heat shock, alkaline pH, oxidative stress and genotoxic stress) (Hedbacker and Carlson, 2008).

In mammals, AMPK (AMP-activated protein kinase) is involved in the sensing of energy level. As its name indicates, this complex is allosterically activated by AMP, effect that is antagonized by high concentrations of ATP (Carling et al., 1994; Hardie et al., 1999). As a result of the reaction catalyzed by adenylate kinase (2ADP↔ ATP+AMP), the AMP:ATP ratio varies approximately as the square of the ADP:ATP ratio, making the former ratio a sensitive indicator of reduced cellular energy status (Hardie and Hawley, 2001). Consequently, any cellular or metabolic stress that inhibits the ATP synthesis, as glucose deprivation, pathological stresses (hypoxia, ischemia, oxidative damage), exercise and dietary hormones (Hardie et al., 2003; Hardie, 2007; O'Neill et al., 2011), or that accelerates the ATP consumption (contraction of skeletal muscles) (Winder and Hardie, 1996) causes AMPK activation. Once activated, AMPK regulates various cellular metabolic processes, acting to downregulate ATP-consumption (e.g. fatty acids and proteins synthesis) and upregulate ATP-production (e.g. fatty acids oxidation, glucose uptake and glycolysis) (Hardie et al., 1999; Woods et al., 2003a; Hardie, 2004).

In plants, SnRK1 is considered a central regulator in response to multiple types of stress (Baena-Gonzalez et al., 2007). The antisense expression of SnRK1 in potato resulted in the loss of the induction of sucrose synthase gene expression (Purcell et al., 1998); and in spinach SnRK1 was found to inactivate *in vitro* sucrose phosphate synthase (Sugden et al., 1999b). In addition, antisense expression of a barley SnRK1 sequence resulted in the arrest of pollen development, producing male sterile plants (Zhang et al., 2001). It is thought that this arrest results from the inability of the pollen to respond to their carbon status (Buitink et al., 2004). *Arabidopsis* plants overexpressing SnRK1.1 are more tolerant to nutrient-deprivation and silencing of SnRK1.1/SnRK1.2 precludes target genes induction upon stress (Baena-Gonzalez et al., 2007). These data demonstrates that SnRK1 regulates metabolism and transcription in response to energy deprivation, caused by nutrient deprivation, environmental stress or alternate light-dark cycle. Once activated, SnRK1 triggers changes in the gene expression to repress energy consuming processes and promote catabolism, in order to re-establish the energy homeostasis (Thelander et al., 2004; Schwachtje et al., 2006; Polge and Thomas, 2007; Lee et al., 2009). When the sugars balance is restored, SnRK1.1 is inactivated in a process that might involve trehalose-6-phosphate (Baena-Gonzalez et al., 2007; Zhang et al., 2009). SnRK1 also regulates aspects of cell function and plant development. Similarly to their orthologs, SnRK1 regulates cell cycle progression and is involved in specific plant processes from germination until senescence, such as seed filling and maturation, embryo development, cotyledon growth and sprouting, pollen development and male sterility, lateral organ development and phase transition (Zhang et al., 2001; Radchuk et al., 2006; Radchuk et al., 2010; Tsai and Gazzarrini, 2012; Guerinier et al., 2013). In addition, this protein is also involved in antiviral defense (Hao et al., 2003).

As mentioned, SnRK1 regulates metabolism and gene transcription, through modulation of enzymes activity by phosphorylation or redox activation of metabolic enzymes, and activation/inactivation and control of recruitment and localization of various transcription factors (Halford and Hey, 2009). SnRK1 controls by inactivation through phosphorylation various enzymes such as, 3-hydroxy-3-methylglutarylcoenzyme A reductase (HMG-CoA reductase: sterol biosynthesis), sucrose phosphate synthase (SPS: sucrose synthesis), nitrate reductase (NR: nitrogen assimilation), trehalose-phosphate synthase (TPS: desiccation tolerance), and 6-phosphofructo-2-kinase/fructose-2, 6-

bisphosphatase (F2KP: photosynthate partitioning). SnRK1 also regulates the ADP-glucose pyrophosphorylase through both modulation of its redox state and gene expression, and the enzymes sucrose synthase, α-amylase, and sugar-repressed/dark-induced asparagine synthase that are controlled through gene expression (Coello et al., 2012). SnRK1 is also a global regulator of gene expression involved in primary and secondary metabolism and protein synthesis, and it is believed to controls the activity of transcription factors (Mair et al., 2015). The DIN1 and DIN6 genes are also specifically activated by SnRK1, under diverse stress conditions, limiting the photosynthesis and respiration. Besides these genes, SnRK1 was identified as regulator of a large number of genes encoding chromatin remodeling factors and a plethora of signal transduction components (Baena-Gonzalez et al., 2007). SnRK1.1 overexpression repressed the expression of MYB75/PAP1 (a key transcription factor for anthocyanin biosynthesis) and of bZIP transcription factors (bZIP1, bZIP2, bZIP11, bZIP44, bZIP53 and bZIP63; participating in stress signaling) (Baena-Gonzalez, 2010). Interestingly, bZIP63 was recently identified as target of SnRK1 (Mair et al., 2015). It has also suggested that SnRK1 could regulate stress signaling through the ABA response element binding proteins (AREBPs) that are highly conserved SnRK1 target sites. AREBP is a family of bZIP transcription factors unique to plants, which regulate the ABA responsive genes (Zhang et al., 2008). Moreover, SnRK1 overexpression has an effect on the vegetative and reproductive growth and development transition, which may enable a more targeted genetic modification of plant development, architecture, carbon allocation, and stress resistance (Baena-Gonzalez et al., 2007). Several hormone metabolic pathways and hormone responsive genes expression are also affected by SnRK1 (Baena-Gonzalez and Sheen, 2008).

In plants, glucose affects development processes throughout the plant life cycle, including germination, early seedlings growth, flowering and senescence (Arenas-Huertero et al., 2000; Gibson, 2005). Genetic analysis have shown that sugar signaling establish close crosstalk with plant hormone biosynthesis and signaling (Cao et al., 2006; Dekkers et al., 2008). A large number of mutants with altered sensitivity to sugars were identified through genetic screens. These *Arabidopsis* mutant are able to germinate and grow in the presence of sugar (glucose, mannose or sucrose) concentrations that are inhibitory to wild-type seeds. Their altered response allowed the identification of a large number of sugar-response loci mutations that also cause defects in hormone synthesis or response, revealing interactions between sugar and hormonal signaling pathways (Zhou et al., 1998; Gibson, 2000; Gazzarrini and McCourt, 2001). Some of these mutations are allelic to previously known mutations in ABA synthesis (*aba*) or sensitivity (*abi*) (Finkelstein et al., 2002). Furthermore, additional sugars partially overcame the inhibition of germination by ABA in wild-type and *abi* mutant seeds (Garciarrubio et al., 1997; Finkelstein and Lynch, 2000). Conversely, in response to sugars ABA induced the expression of starch biosynthetic genes, suggesting that ABA can enhance the ability of tissues to respond to sugar signals (Rook et al., 2001). The *sugar-insensitive1* (*sis1*) mutant is allelic to *ctr1*, a mutant with altered response to ethylene that displays multiple phenotypes of resistance to glucose, mannose and to the gibberellin (GA) biosynthetic inhibitor paclobrutazol (PAC) (Gibson et al., 2001). These findings suggest hormonal-sugar signaling pathways interactions and raise the possibility that genes involved in these interactions might be transcriptionally regulated by sugar (Bradford et al., 2003; Leon and Sheen, 2003; Yuan and Wysocka-Diller, 2006)

Organisms frequently modulate signal transduction events by reversible phosphorylation or dephosphorylation. The activation of SNF1, AMPK and SnRK1 depends on their phosphorylation by upstream kinases. These kinases are responsible for the phosphorylation of a conserved threonine (Thr) residue, within the T-loop of the catalytic subunit ($\text{SnRK1}\alpha1^{\text{T175}}$/$\text{SnRK1}\alpha2^{\text{T176}}$; $\text{AMPK}\alpha2^{\text{T172}}$; $\text{SNF1}^{\text{T210}}$). SNF1 is phosphorylated by Sak1, Elm1 and Tos3 kinases. AMPK is phosphorylated by LKB1 and CaMKK. Trough sequence comparison, complementation and phosphorylation assays GRIK1/2 (SnAK1/2) were identified in *A. thaliana* as the upstream activating kinases of SnRK1 (Hawley et al., 2003; Hong et al., 2003; Woods et al., 2003b; Hawley et al., 2005; Woods et al., 2005; Harthill et al., 2006; Shen and Hanley-Bowdoin, 2006; Hey et al., 2007). Conversely, dephosphorylation of the T-loop by upstream phosphatases results in the deactivation of SNF1/AMPK/SnRK1. This regulatory mechanism is better understood in yeast. In response to high glucose signal SNF1 is dephosphorylated by the PP1 phosphatase Glc7, in complex with the regulatory subunit Reg1, resulting in its deactivation (Hedbacker and Carlson, 2008). The importance of this regulatory mechanism is well illustrated by the lethality of the null Glc7 mutant, probably due to an excessive SNF1 activity. Recently it has been shown that SNF1 is also dephosphorylated by the type 2C phosphatase Ptc1 and by the type 2A phosphatase Sit4 and the results suggest that though Glc7-Reg1 is the major phosphatases all three contribute to the maintenance of the Snf1 activation loop in the dephosphorylated state during growth on high glucose (Ruiz et al., 2011, 2013). In the AMPK case, PP1, PP2A and PP2C type phosphatases dephosphorylate *in vitro* this protein kinase, but PP1 and PP2C appears more efficient even *in vivo* (Carling et al., 1989; Davies et al., 1995; Steinberg and Kemp, 2009; Garcia-Haro et al., 2010; Carling et al., 2012).

As opposed to what is observed in yeast and mammals, in plants a clear correlation between SnRK1 inactivation and T-loop dephosphorylation has not been established. However, human PP2C is able to dephosphorylate SnRK1.1 *in vitro* (Sugden et al., 1999a) and two *Arabidopsis* phosphatases, PP2C74 and PTP-KIS1, have also been identified as interactors of SnRK1.1 using the yeast two hybrid system (Niittyla et al., 2006; Koetting et al., 2009; Tsugama et al., 2012) suggesting that dephosphorylation might be an important SnRK1 regulatory mechanism. Recently, it was shown that a least two of the clade A PP2Cs, ABI1 and PP2CA, interact and dephosphorylate SnRK1.1 resulting in its inactivation (Rodrigues et al., 2013), also supporting a regulatory role for the SnRK1 T-loop dephosphorylation.

PP2Cs are key repressors of the ABA signaling pathway (Cutler et al., 2010). Under basal ABA levels (in the absence of stress), PP2Cs act as constitutive negative regulators of SnRK2s, which require ABA-induced phosphorylation to activate downstream targets (Fujii and Zhu, 2009). When ABA levels rise (in response to stress), PYR/PYL/RCAR ABA-receptors inactivate the PP2Cs (Ma et al., 2009; Park et al., 2009), which allows activation of the SnRK2s, subsequent phosphorylation of transcription factors and transcriptional response to ABA (Kobayashi et al., 2005; Fujii et al., 2009). In this way, PP2Cs established a link between the energy and ABA signaling pathways and potentiate a coordinated response to stress conditions.

An Y2H (Yeast Two Hybrid) screening identified the interaction between SnRK1.1 and NDF4 (NDH-dependent cyclic electron flow) (Azinheiro, 2015). NDF4 is a subunit of the NDH (NAD(P)H dehydrogenase) complex localized in chloroplast, that is involved in the photosynthesis, specifically in the PSI (photosystem I) cyclic electron flow. NDH complex protect against over reduction and control the ATP/NADPH ratio. The cyclic electron flow is also controlled by the Proton Gradient Regulation 5 (PGR5)-dependent pathway (Munekage et al., 2002; Munekaga et al., 2004). The PGR5 pathway regulate the mechanism in normal physiological conditions, NDH-dependent pathway regulate in photo-oxidative stress conditions (Takabayashi et al., 2002; Munne-Bosch et al., 2005; Wang et al., 2006). Moreover, NDH also supplies excess ATP for CO_2 fixation through C_4 cycle and function in chlororespiration (Peltier and Cournac, 2002; Takabayashi et al., 2005).

A high-troughput screening identified Repressor of GA (RGA) and Gibberellic Acid Insensitive (GAI) as NDF4 interactors (Braun et al., 2011). RGA and GAI belong to a group of proteins known as DELLA, named after the presence of highly conserved five amino acids domain in the N-terminal region. The DELLA proteins are key components of the gibberellin signaling pathway.

Gibberellins (GA) are a class of plant hormone involved in multiple development processes, including seed germination, stem elongation, leaf expansion, trichome development, pollen maturation, induction of flowering and fruit growth (Hooley, 1994; Phillips, 1998; Davies, 2004). *Arabidopsis* mutant plants deficient in GA synthesis, *ga1-3* (do not produce an important enzyme of GA biosynthesis pathway), fail in germination, are dwarf and exhibit dark green leaves, late flowering phenotype and are male sterile. These phenotypes are restored with exogenous GA treatment (Koornneef and Vanderveen, 1980; Wilson et al., 1992; Silverstone et al., 2001).

In the past decade, genetic screenings in rice and *A. thaliana* led to the identification of the three core elements of the GA signaling pathway, the nuclear GA-receptor (GID1), the DELLA proteins, and the F-BOX proteins (Ueguchi-Tanaka et al., 2008). GID1 (GA INSESITIVE DWARF 1) is the element that interact with active gibberellins. In rice there is only one gene coding for the GID1 receptor but in *Arabidopsis* there are homologs, GID1a, GID1b and GID1c (Ueguchi-Tanaka et al., 2005; Griffiths et al., 2006; Nakajima et al., 2006). DELLA proteins are negative regulators of GA signaling (Peng et al., 1997; Silverstone et al., 1998; Dill and Sun, 2001). The F-box protein is a subunit SCF E3 ubiquitinase complex, which is involved in DELLA proteins degradation - in rice is the GA-INSENSITIVE DWARF2 (GID2) and in *Arabidopsis* are the SLEEPY1 (SLY1) and SNEEZY (SNZ) (McGinnis et al., 2003; Sasaki et al., 2003).

The actual model to explain the gibberellins pathway proposes that GA is detected and bind to GID1, forming the complex GA-GID1. This link, at the Tyr 31 residue of GID1, induces a conformational change that allows and enhance the interaction with the DELLA proteins, in DELLA and TVHYNP regions, forming the GA-GID1-DELLA complex (Griffiths et al., 2006; Murase et al., 2008; Shimada et al., 2008). The formation of this complex led to a conformational change at the C-terminal of DELLA proteins, which enhance the recognition between the VHIID and LHTII regions and the F-Box SLY/GID2. The SCF$^{SLY/GID2}$ complex (SKP1-CULLIN1-F-BOX) polyubiquitinates the DELLA proteins, targeting them for destruction by the 26S proteasome (Dill et al., 2001; McGinnis et al., 2003; Sasaki et al., 2003; Fu et al., 2004; Hirano et al., 2010). So, when GA is in absence, DELLA proteins accumulate in the nucleus, interact with transcription factors of GA-responsive genes and restrain growth and development processes; when GA is present, DELLA proteins are degraded, overcoming DELLA-mediated growth restrain (Fig. 1.3) (Hirsch and Oldroyd, 2009; Mutasa-Goettgens and Hedden, 2009).

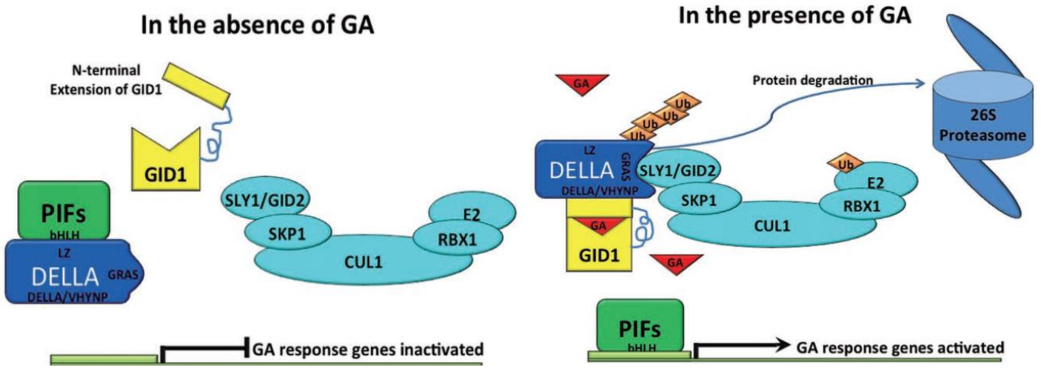


Figure 1.3– Model of protein interaction cascade in GA signaling.
In the absence of GA, GID1 do not form complex with DELLA proteins, allowing the repression of GA-response. In the presence of GA, the formation of the GA-GID1-DELLA complex recruit SCF$^{SLY1/GID2}$, targeting DELLA for ubiquitination and consequent degradation, overcoming DELLA-mediated growth restrain (Wang and Deng, 2011).

DELLA proteins are an evolutionally conserved proteins that act as negative regulators of GA-signaling and are localize in plant cell nuclei. Based on sequence similarities, DELLA proteins are categorized as a subfamily of the GRAS proteins family. GRAS proteins is a superfamily of putative transcription factors unique in plants. The sequence alignment of all GRAS proteins reveals a common homology in some domains of the C-terminal region such as, two leucine heptad repeats (LHR I and LHR II, which may mediate protein-protein interaction), VHIID, PFYRE and SAW. These motifs are involved in transcriptional regulation (Pysh et al., 1999). On the other hand, the N-terminal of GRAS proteins is highly divergent, probably due to the specific roles in different cellular pathways. The N-terminal of DELLA proteins contains three conserved domains, DELLA, TVHYNP and the poly-S/T/V (serine/threonine) stretch (Fig. 1.4) (Dill et al., 2001; Gubler et al., 2002; Itoh et al., 2002; Olszewski et al., 2002; Sun and Gubler, 2004). The domains close to N-terminal not only differentiate the DELLA proteins from other GRAS proteins, but have also an important role in the GA signaling. The semi-dominant *gai-1* allele contains a 51-bp (base pair) deletion in the region of the DELLA domain, including the 17-amino acid residues segment-DELLAVLGYKVRSSEMA (Koornneef et al., 1985; Peng et al., 1997). This mutant shows a semi-dwarf phenotype, like *ga1-3* mutants, but in this case the exogenous GA treatment cannot rescue the phenotype. It was demonstrated that these 17 amino acid residues are responsible for the interaction with GA, and their deletion makes *gai-1* into a gain-of-function repressor of GA signal (Peng et al., 1997). The same deletion in RGA and RGL2 (Dill et al., 2001; Hussain et al., 2005) and in the RGA and GAI orthologs (*Rht*, *d8* and *SLR*) leads to a GA-insensitive dwarf phenotype, in the respective species (Peng et al., 1999; Ikeda et al., 2001).

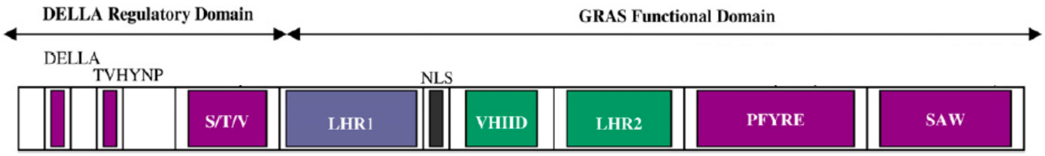


Figure 1.4 – Schematic representation of DELLA proteins.
The conserved motifs involved in GID1 binding (purple), protein-protein binding (blue), nuclear localization (grey) and SLY binding (green). Also are represented over-lines the divisions of DELLA domain (DELLA regulatory domain) and GRAS domain (GRAS functional domain) (Hauvermale et al., 2012).

Genes encoding DELLA proteins have been isolated in diverse plant species. The *A. thaliana* genome encodes five DELLA proteins, GA-INSENSITIVE (GAI), REPRESSOR OF GA 1-3 (RGA), and RGA-LIKE1, 2 and 3 (RGL1, RGL2 and RGL3). The other plants genomes encode single DELLA protein, SLENDER RICE1(SLR1) in rice, SLENDER1 (SLN1) in barley, DWARF8 (D8) in maize, REDUCED HEIGHT (Rht) in wheat and VITIS VINIFERA GAI (VvGAI) in grape (Peng et al., 1997; Silverstone et al., 1998; Peng et al., 1999; Ikeda et al., 2001; Boss and Thomas, 2002; Chandler et al., 2002; Lee et al., 2002; Wen and Chang, 2002).

In the *Arabidopsis* genome, GAI and RGA proteins have 82% of similarity in their amino acid sequence (Silverstone et al., 1998). These two proteins are important in the control of stem elongation, as was demonstrated in a double null alleles mutant (*gai-t6* and *rga-24*), which suppress the stem elongation, shoot and root growth phenotype conferred by the *ga1-3* mutation (Peng et al., 1997; Silverstone et al., 1998). However, experiments testing the effect of loss-of-function of GAI or RGA resulted in a partial reduction of phenotype exhibited by *ga1-3*, wherein the loss of RGA had more obvious effects than loss of GAI. This demonstrated that RGA and GAI interact synergistically in repressing GA signaling, despite the greater contribution of RGA than GAI (Dill and Sun, 2001). The analysis of other plant parts shown that double mutant develop abnormal flowers and requires exogenous GA to germinate, similar to *ga1-3* phenotypes. These analysis suggested that RGA and GAI cannot regulate germination or flower development, it means that the other open reading frames (ORFs) with high degree of sequence similarity to GAI and RGA (RGL1, RGL2 and RGL3), may regulate flowering and germination to GA responses (Dill and Sun, 2001). RGL1, RGL2 and RGL3 genes exhibit 56% to 60% of similarity to GAI and RGA, including the domains close to C-terminal and the domains close to N-terminal (Lee et al., 2002). The presence of the two domains DELLA and TVHYNP in the N-terminal led to the hypothesis that the three RGLs encode also negative regulators of GA signaling pathway, regulating GA responses which are not dramatically affected by RGA or GAI (Dill and Sun, 2001; Lee et al., 2002). Experimental data shown that *rgl2-1* confers resistance to the inhibitory effect of PAC on seed germination, and double null allele mutants *rgl2-1* and *ga1-3* achieved approximately 95% germination (opposite phenotype to the *ga1-3*), showing that RGL2 is the major protein responsible for controlling the germination (Lee et al., 2002; Cao et al., 2005). Cheng et al. (2004) shown that RGL1 and RGL2 are involved in petal and stamen development and have a minor role in stem elongation (Cheng et al., 2004). RGL3 is less known but is thought to be involved in floral development with the three others and in germination (Tyler et al., 2004). Analysis of gene expression of DELLA proteins and phenotypic analysis of *dellaKO* mutants in *Arabidopsis* have indicated the specific role and the degree of redundancy (Silverstone et al., 1997; Dill and Sun, 2001; Lee et al., 2002; Cheng et al., 2004; Tyler et al., 2004). In rice and wheat, the redundancy is not visible due to the single DELLA protein (Ikeda et al., 2001; Chandler et al., 2002).

During the GA-mediated growth, DELLA proteins degradation is an important step of the GA signaling pathway. Biochemical studies of yeast and mammals have shown that the interaction of F-box proteins with protein substrates is dependent of modifications, including phosphorylation (Achard and Genschik, 2009). Phosphorylation and dephosphorylation of proteins is a very important regulatory mechanism because it can change the activity and/or stability of proteins. In rice, the initial reports suggested that the DELLA proteins were phosphorylated by an unknown kinase, linked to GID2, promoting their degradation by the proteasome 26S (Sasaki et al., 2003). Similar results were obtained in *Arabidopsis*, for DELLA protein GAI (Fu et al., 2004). However, an increasing number of results suggest that phosphorylation of the DELLA proteins increase their stability. RGL2 degradation induced by GA is blocked by serine/threonine phosphatase inhibitors; however, serine/threonine kinase inhibitors had no detectable effect suggesting that dephosphorylation is probably required for RGL2 degradation (Hussain et al., 2005). In addition, PP1/PP2A phosphatase inhibitors block DELLA degradation indicating that degradation of DELLA proteins required protein Ser/Thr dephosphorylation activity (Wang et al., 2009). Supporting the role of phosphorylation in the stabilization of the DELLA protein it has recently been showed that phosphorylation of the rice DELLA protein SLR1 in specific Ser/Thr residues is important for maintaining its stability, and that mutation of the candidate phosphorylation residues resulted in the altered GA signaling (Dai and Xue, 2010). A possible explanations for the first opposite role suggested might be related to the observation that Tyr kinase inhibitors but not phosphor-Tyr phosphatase inhibitors block GA-induced degradation of RGL2 protein (Hussain et al., 2007). This result might suggest that phosphorylation of specific Tyr residues would contribute to the DELLA degradation while the phosphorylation of specific Ser/Thr residues would have the opposite effect.

MATERIALS AND METHODS

2. Materials and methods

2.1.Proteins interaction

2.1.1. Plasmids construction

To test the interaction between SnRK1.1 and the different DELLA proteins (full length and deletions), in the presence and absence of NDF4, the yeast two hybrid (Y2H) and the pull-down methods were used.

The coding sequence of the DELLA proteins (full length and deletions) and of NDF4 were PCR-amplified with specific primers (table I) and using cDNA obtained by reverse transcription of RNA extracted from mature leaves of *Arabidopsis* plants. The PCR reactions were performed with Q5 DNA polymerase (NEB-New England BioLabs) according to the manufacturer's instructions, using 30-cycles and an annealing temperature of 55°C, except for GAI in which 60°C was used. The PCR-amplifications were confirmed by agarose electrophoresis gel with 5µL of the reaction, and the remaining PCR products were purified by phenol-chloroform. It was added sterile water up to 200µL and the same volume of phenol-chloroform (50:50). This mixture was vortexed for 1 minute and centrifuged to separate the phases. The aqueous phase was recovered and the DNA was precipitated with 3 volumes of ethanol 100% and 1/10 volume of NaAc 3M pH=5.2, followed by incubation at -20ºC, for 30 minutes. After centrifugation, 15000g for 15 minutes, the supernatant was rejected, and the pellet was washed with 1ml of ethanol 70%. After dying, the pellet was resuspended in 12 µL of water. Once purified, the PCR products were digested with the respective restriction enzymes (NEB), NDF4, GAI, RGA, RGAΔC and RGAΔN with BamHI and SalI, GAIΔC and GAIΔN with BamHI and EcoRI. The pET28 and pGADT7 plasmids were digested with the same restriction enzymes. The restriction products were separated by agarose electrophoresis gel and the required bands were excised from the gel. The DNAs of the excised gel fragments were extracted from the agarose gel using the Gel Extraction kit (QIAGEN). The purified and digested DNA fragments were cloned in the respective vectors using T4 ligase (NEB), according to the manufacturer's instructions. NDF4, GAI and RGA were cloned into pET28 for the pull down test, GAIΔC/ΔN and RGAΔC/ΔN were cloned in pGADT7 for the Y2H test. These ligation products were used to transform competent *E. coli* cells (TOP10 strain) by heat shock. Transformed cells were plated in solid LB medium with antibiotic (ampicillin for pGADT7 and kanamycin for pET28) and incubated at 37°C for 12h-16h. After incubation, the colonies were peaked to liquid lysogeny broth (LB) medium with the respective antibiotic and incubated during at 37°C for 12h-16h. The plasmids were extracted using ZR Plasmid Miniprep-Classic kit (ZYMO RESEARCH). The correct binding of DNA fragments in vectors was tested by digestion with the same or specific restriction enzymes followed by the analysis of the restriction fragments by agarose electrophoresis gel. The SnRK1.1 constructions in pBridge (pBridge-SnRK1.1-NDF4) were previously described (Azinheiro, 2015).

Table 2.1 - Primers used to amplifying the coding sequence of the genes to be cloned.
In lowercase 3 bases (ttt or aaa) followed by the recognition sequence of the restriction enzyme used.

Gene	Primer sequence 5'-3'
NDF4 BamHI Fw	tttggatccATGGGAAGTGTACAGTTGAGT
NDF4 Sal Rev	tttgtcgacTCAAGTAGAAGTTTCGAGATCA
GAI BamHI Fw	tttggatccATGAAGAGAGATCATCATCA
GAI Sal Rev	tttgtcgacCTAATTGGTGGAGAGTTTCCAAG
GAI ΔC BamHI Rev	tttggatccAGTATCGGAGAGAGAGTGGT
GAI ΔN EcoRI Fw	aaagaattcCTTCAGATGCACTTCTACGAG
RGA BamHI Fw	tttggatccATGAAGAGAGATCATCACCAAT
RGA Sal Rev	tttgtcgacTCAGTACGCCGCCGTCGAGA
RGA ΔC SalI Rev	tttgtcgacAGTATCGGAGAGACAATGATCG
RGA ΔN BamHI Fw	aaaggatccATCTTCAGATGCACTTTTACGAG

2.1.2. Yeast two hybrid (Y2H) screening

The cells of *S. Cerevisiae* Gold strain (Clontech) were cotransformed by the lithium acetate method (Gietz et al., 1997). Briefly, 1µg of each plasmid (pGADT7-GAI/RGA/RGL or pGADT7-GAI/RGAΔC/ΔN and pBridge-SnRK1.1 or pBridge-SnRK1.1-NDF4) was added to 25-50 µL of competent yeast cells. Next, 800µL PEG-LI-

TE at 40% was added and the cells were incubated during at 30°C 30 minutes, followed by an incubation at 42°C during 20 minutes. After centrifugation during 5 minutes at 2000g the supernatant was eliminated and the cells were resuspended in 100µL of sterile water and plated in SD medium (Clontech) without leucine (L) and tryptophan (W) but with adenine (A) and histidine (H) (medium SD-L-W) to select the colonies that were cotransformed with both plasmids. The plates were incubated during two days at 30°C and three colonies randomly selected were used to inoculate SD-L-W liquid medium. These cultures were used to do dilutions (10^{-1}; 10^{-2}; 10^{-3}), that were spotted on selective medium-SD without leucine, tryptophan, adenine (A) and histidine (H), medium (SD-L-W-A-H), and incubated during two or three days at 30°C to test interaction. The interaction of SnRK1.1 and the DELLA proteins deletion was also tested in less stringent medium, SD-L-W-H with or without aminotriazole (30mM). The same dilutions were also spotted in SD-L-W medium as a positive control. Yeast cells were also cotransformed with pGBKT7-empty and pGADT7-DELLA (full length or deletions) as negative control to confirm that DELLA proteins are no able by themselves to activate the system (background).

2.1.3. Recombinant protein production

The coding sequence of RGA, GAI and NDF4 were cloned into pET28 to generate a HIS-Y7-Protein fusion. Recombinant proteins were produced in *E.coli* (BL21) and purified using IMAC (TALON, Clontech) following manufacturer's instructions.

Recombinant SnRK1.1-GST was produced in E.coli (BL21:DE3) and purified through GSH affinity chromatography using GSH-agarose beads as recommended by the manufacturer (Sigma G4510). Successful protein production and purification were verified by immunoblotting with anti-GST and anti-T7 antibodies.

2.1.4. *In Vitro* Pull down assay

Proteins (3µg of each) were incubated 1h at room temperature in 100µL of interaction buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 0.05% Triton-X100, 1/500 (V/V) plant-specific protease inhibitor cocktail (Sigma P9599), mixed with 30 µL of GSH-agarose beads (Sigma G4510) and incubated one extra hour at room temperature. Beads were washed 4 times with interaction buffer, and bound proteins were analyzed by Western blotting using anti-T7 antibodies.

2.2.*In vivo* tests

2.2.1. Plant material

All phenotype assays were realized with wild type (Col-0) seeds and SnRK1.1 overexpressor mutant seeds (SnRK1.1 OX) in Col-0 background (Jossier et al., 2009). For *in vitro* culture, seeds were surface-sterilized ,with a solution containing ethanol 70%, 0,05% Triton X-100, for 10 minutes, washed five times with sterile water and stratified in the dark at 4°C for 2 days. Seeds were sown on Murashige-Skoog (Duchefa) medium plates with 0,8% of phytoagar (Duchefa), 1% of sucrose (Sigma), 0,05% MES (Sigma). The pH was adjusted to 5.7/5.8 with KOH, before autoclaving. The medium was supplemented with the hormones, gibberellins (GA_3; Sigma) or abcisic acid (ABA; Sigma) or GA biosynthesis inhibitor paclobutrazol (PAC; Sigma), as specified below. Plates were sealed and incubated at 23°C under a 12h-light/12h-dark regime in controlled environment conditions.

2.2.2. Germination test

For germination assay, seeds were surface-sterilized and stratified as described and sown in MS medium supplemented or not supplemented with GA_3 (1µM), PAC (2µM) or ABA (1µM). Seeds with radicle emergence were counted 1 day after sowing in MS, GA_3 and PAC conditions, and 3 days after sowing in ABA condition. The experience was repeated three times on four plates with about 50 seeds of each genotype and condition. Values are averages ± SD for three independent experiments. Statistical analysis were calculated with Student's t test, with a confidence level of 95% ($P<0,05$), when comparing data from SnRK1 OX and WT in the same assay conditions.

2.2.3. Hypocotyl growth test

For hypocotyl growth assay, seeds were sown in MS medium supplemented or not with GA_3 (1µM) and plates were incubated in vertical position. Seedlings were photographed after 7 days with a digital camera and the hypocotyl length was measured with the ImageJ software (Schneider et al., 2012). The experiences were repeated three times on five plates with about 6 seeds of each genotype and condition. Values are averages ± SD for three independent experiments. Statistical analysis were calculated with Student's t test, with a confidence level of 95% ($P<0{,}05$), when comparing data from SnRK1 OX and WT in the same assay conditions.

2.2.4. Flowering preliminary-assays

For flowering assays, seeds were sown in MS medium and the plates were incubated in vertical position. After five days seedlings were transferred to MS medium supplemented or not with GA_3 (1µM). The plants were photographed 21 days after sowing. The experience was repeated one time on three plates with 5 or 6 seedlings of each genotype and condition.

RESULTS

3. Results

3.1.SnRK1.1 interacts with the DELLA proteins

The interaction between SnRK1.1 and the DELLA proteins in the presence of NDF4 using the Y2H was previously observed (Azinheiro, 2015) to further study this interaction we decide to analyze the importance of the presence of NDF4, also using the Y2H system

The results show that in Y2H SnRK1.1 interacted with the DELLA proteins both in the presence or absence of NDF4 (Fig. 3.1-A). However, yeasts growth was slower in the absence of NDF4 suggesting that NDF4 could be important for the interaction.

To further validate the results observed using the Y2H system, *in vitro* pull down assays using recombinant proteins were performed. Purified recombinant Hist-T7-RGA and Hist-T7-GAI was incubated in the presence or absence of Hist-T7-NDF4 and with GST-SnRK1.1 or GST and the interacting proteins were pulled down using a glutathione-agarose matrix. RGA and GAI were recovered in the presence and absence of NDF4 (Fig. 3.1-B), confirming the interaction between SnRK1.1 and the DELLA proteins. Interestingly, in the presence of NDF4, the band intensity of RGA/GAI is lower than in its absence, suggesting that NDF4 might not be important for SnRK1.1-DELLA proteins interaction. This decrease of the DELLA bands intensity might indicate that NDF4 and RGA/GAI compete for biding to SnRK1.1.

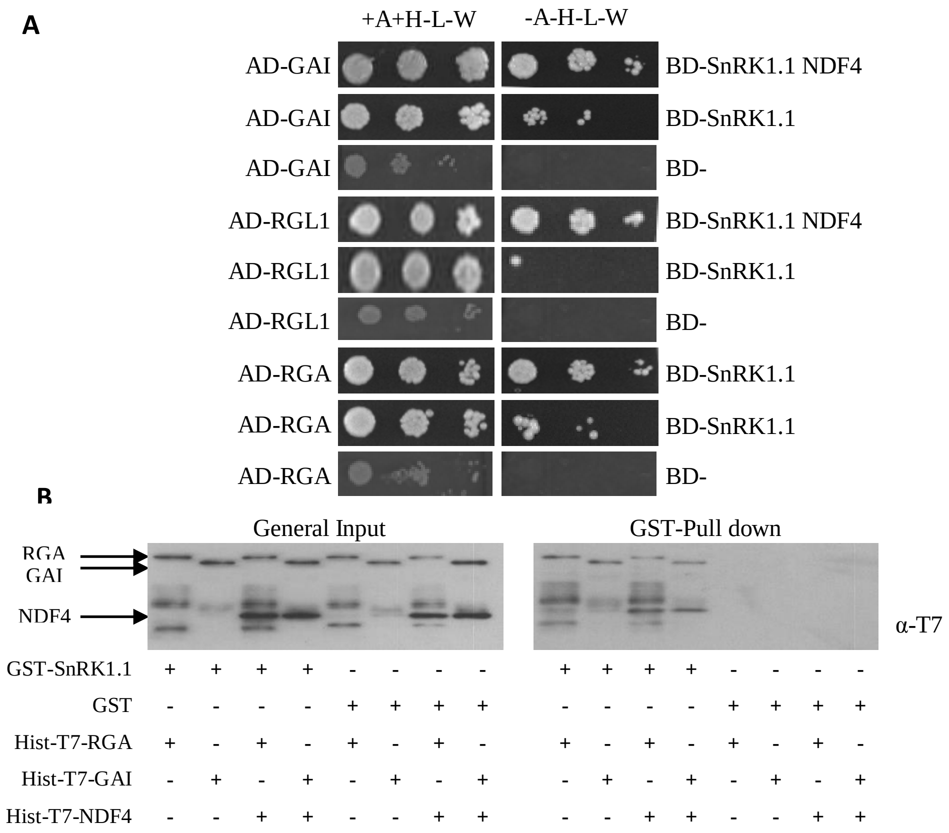


Figure 3.1 - SnRK1.1 interacts with DELLA proteins *in vivo* and *in vitro*.
A- Using the Y2H system, SnRK1.1 interacts with the DELLA proteins (full length) in presence and absence of NDF4. Protein interaction was determined by growth of yeast cells in three successive dilutions (10^{-1}, 10^{-2}, 10^{-3}) of saturated cultures, in medium lacking Leu, Trp, adenine and Hist (-L-W-A-H) compared with control medium lacking Leu and Trp but supplemented with Adenine and Hist (-L-W+A+H). **B-** *In vitro* interaction between DELLA proteins (RGA and GAI), NDF4 and SnRK1.1-GST detected by GST pulldown and T7 immunodetection of SnRK1.1.

To further study the interaction between SnRK1.1 and DELLA, we decide to map this interaction (Fig. 3.2) using ΔC deletions of GAI and RGA containing the DELLA, TVHYNP and poly-S/T/V motifs, and ΔN deletions containing the LHR, NLS, VHIID, RFYRE and SAW motifs. SnRK1.1 did not interact with these DELLA deletions in the -L-W-A-H selective medium (Fig. 3.2). Interestingly, using a less stringent medium +A-H-L-W+ aminotriazole (AT) (30mM), a week interaction between RGA ΔC and SnRK1.1 was observed (Fig. 3.2). Further decreasing the medium stringency (+A-H-L-W) yeast growth was observed using all deletions, but yeasts with RGA/GAI ΔC grown a little more than yeasts with RGA/GAI ΔN. In this conditions the presence or absence of NDF4 didn't seem to have any influence in the growth of the yeast cells, suggesting that NDF4 is not important for the interaction. These results might indicate that independently of the NDF4 presence, both domains of DELLA proteins are important to the interaction with SnRK1.1, although motifs close to N-terminal have more relevance in the interaction with the kinase.

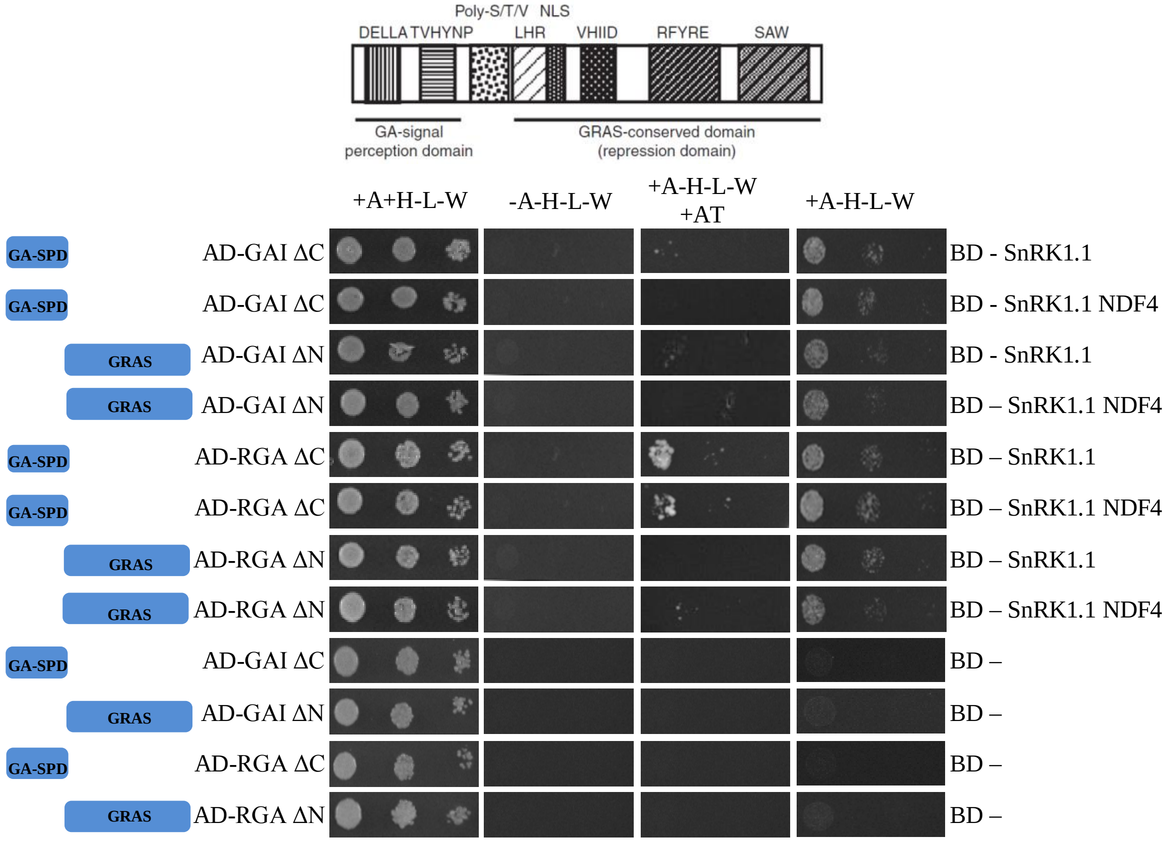


Figure 3.2 - Mapping of SnRK1.1 interacts with the DELLA proteins.
RGA ΔC and RGA/GAI ΔN interact with SnRK1.1 in the Y2H system. Protein interaction was determined by growth assay of three successive dilutions (10^{-1}, 10^{-2}, 10^{-3}) of a saturated cultures, in medium lacking leucine, tryptophan, adenine and histidine (-L-W-A-H) on column 2; medium lacking leucine, tryptophan and histidine, but supplemented with adenine and with aminotriazole (AT, at 30mM) (-L-W+A-H+AT) on column 3; in medium lacking leucine, tryptophan and histidine, but supplemented with adenine (-L-W+A-H) on column 4, compared with control medium lacking leucine and tryptophan but with presence of adenine and histidine (-L-W+A+H). *GA-signal perception domain (GA-SPD); GRAS-conserved domain (GRAS).

3.2.SnRK1.1 overexpression mutants are insensitive to GA

Taking in account that SnRK1.1 interacts with the DELLA proteins and the function of these proteins we hypothesized that this interaction might have a relevant role in the gibberellin signaling pathway. Thus, to address the physiological role of the interaction between SnRK1.1 and the DELLA proteins we analyzed the responses to GA of SnRK1.1 overexpressing mutants (SnRK1 OX) compared to WT.

3.2.1. Germination

GAs have an important role in promoting germination. In the conditions used, the germination rate in MS is closed to 100%, so adding exogenous GA had little or no effect (Fig. 3.3). On the contrary, the addition of the GA biosynthesis inhibitor paclobrutazol (PAC) had a clear negative effect in seed germination: it decreased to approximately 56% in WT, and to approximately 29% in SnRK1 OX.

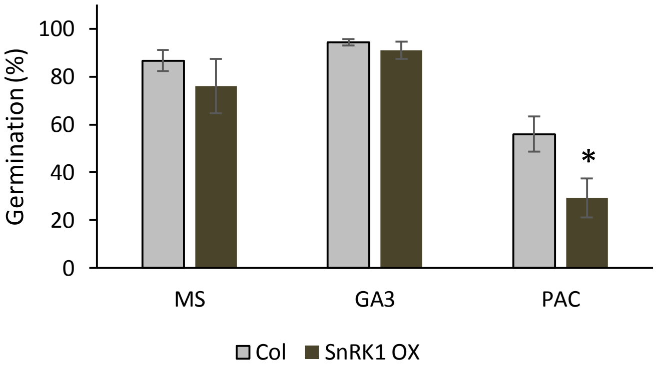


Figure 3.3– SnRK1 OX mutants are hypersensitive to PAC in germination.
Effect of GA_3 (1 µM) and PAC (2µM) in germination. Seeds with radicle emergence of 1mm or more, one day after sowing were counted as germinated. Values are averages ± SD for three independent experiments. *P<0,05 (Student's t test) when comparing data from SnRK1 OX and WT in the same assay conditions.

Abscisic acid (ABA) is an antagonist of GA in germination and so we decide to analyze the influence of this hormone in the germination of Col and SnRK1 OX. The results demonstrate a clear negative effect of ABA on germination being this effect much more pronounced in the SnRK1 OX mutant than in WT (Fig. 3.4). Taken together these results suggest that SnRK1.1 OX mutant is hypersensitive to PAC and ABA.

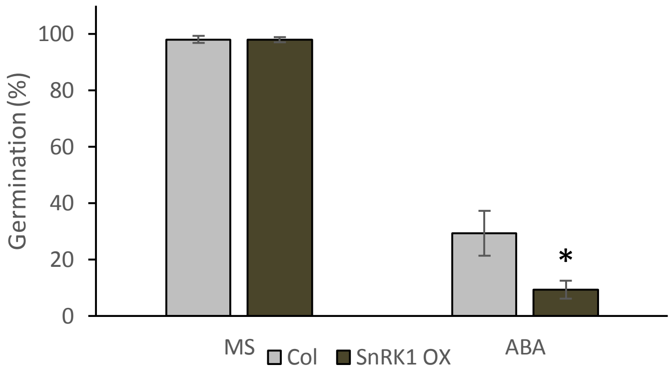
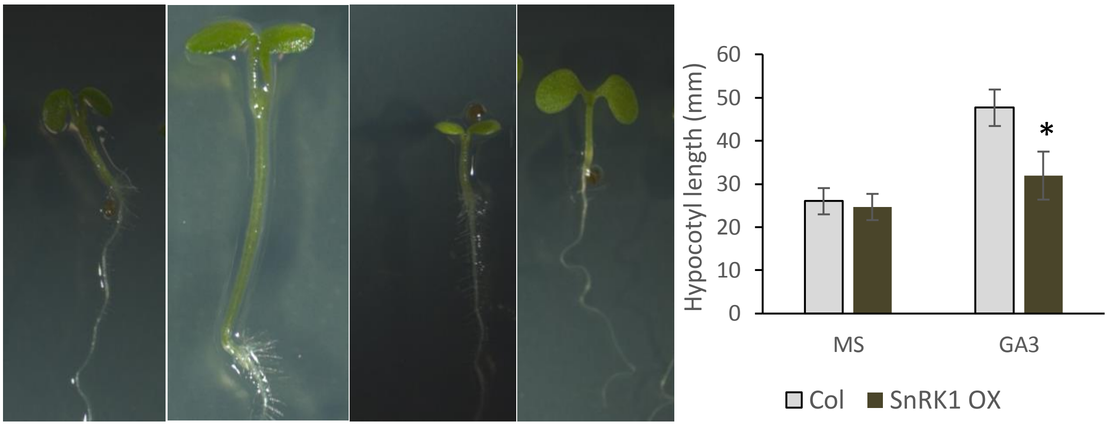


Figure 3.4 - SnRK1 OX mutants are hypersensitive to ABA in germination.
Effect of ABA (1µM) in germination. Seeds with radicle emergence of 1mm or more, three days after sowing were counted as germinated. Values are averages ± SD for three independent experiments. *P<0,05 (Student's t test) when comparing data from SnRK1 OX and WT in the same assay conditions.

3.2.2. Hypocotyl growth assay

Gibberellins play a key role in the process of stem elongation/hypocotyl growth, so we analyzed the influence that SnRK1 overexpression might have in this process, in the presence and absence of GA_3. In MS there is no significant differences in hypocotyl length between SnRK1 OX and WT seedlings (Fig. 3.5). However, in medium supplemented with GA_3 (1µM) the hypocotyl length of WT seedlings is much higher than those of SnRK1 OX (48mm and 32mm, respectively) (Fig. 3.5). This result indicates that SnRK1 OX mutant is insensitive to GA_3 in hypocotyl growth.



WT in MS **WT in MS+GA$_3$** **OX in MS** **OX in MS+GA$_3$**

Figure 3.5 - SnRK1 OX mutants are insensitive to GA$_3$ in hypocotyl growth.
Effect of GA$_3$ (1µM) in hypocotyl length. Hypocotyls were measured 7 days after sowing. Values are averages ± SD for three independent experiments. *P<0,05 (Student's t test) when comparing data from SnRK1 OX and WT in the same assay conditions.

3.2.3. Flowering preliminary-assay

Gibberellins are known to promote flowering. To analyze the effect of SnRK1.1 overexpression in this process, seedlings of SnRK1 OX and WT were kept in MS medium supplemented or not with GA$_3$ (1µL) for 21 days. The results evidentiate a clear effect of GA$_3$ in the flowering time. Only plants in GA$_3$ showed flowers and WT plants flowered earlier than SnRK1 OX plants (Fig. 3.6), indicating that SnRK1 OX are insensitive to GA$_3$.

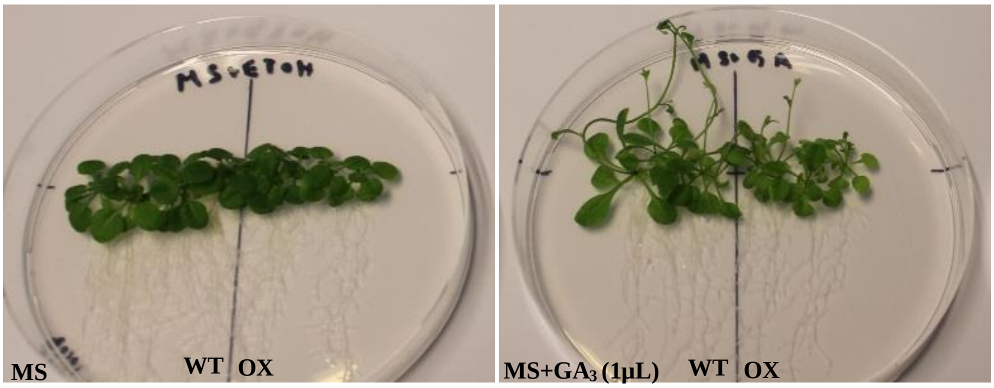


Figure 3.6 - SnRK1 OX mutants show reduced sensitivity to GA$_3$-mediated induction of flowering.
Effect of GA$_3$ (1µM) in induction of flowering. Pictures were taken 21 days after sowing.

DISCUSSION AND CONCLUSIONS

4. Discussion and Conclusions

Despite the importance of the SnRk1 complex in the response to stress and in plant development the molecular mechanisms involved are largely unknown. In this work we uncover SnRK1 as novel component of the gibberellin signaling pathway.

The results obtained using the Y2H and pull down assays indicated that SnRK1.1 interacts with the DELLA proteins both *in vitro* and *in vivo* (Fig. 3.1-A; 3.1-B). The importance of NDF4 in this interaction was not clear, which some contradictory results but in general it seems that NDF4 is not necessary for the interaction between SnRK1.1 and the DELLA proteins. The interaction between NDF4 and SnRK1.1 is clear but its physiological role, if any, is unknown and its cellular co-localization it’s not established. NDF4 is localized in the chloroplast membrane (Munekage et al., 2002) a localization that is not established for SnRK1.1. Interestingly, gibberellins promoted the biogenesis of chloroplasts and as repressors of GA-responses, the five DELLA proteins, but mainly RGA and GAI, repress the chloroplast biogenesis through the repression of the transcription factors AtPDV1, AtPDV2, AtARC5, and AtFtsZ2-1 (Jiang et al., 2012). This observation rises the possibility that SnRK1.1 might have a repressive effect in the chloroplast biogenesis, through its interaction with the DELLA proteins.

Recently, the involvement of the DELLA proteins in the regulation of the photosynthetic machinery was suggested. The DELLA proteins upregulate a photoprotective enzyme in the dark, the protochlorophyllide oxiredutase (POR), protecting against photooxidative damage (Cheminant et al., 2011). The NDH complex, which NDF4 belongs, is also involved in this protection process (Wang et al., 2006). Besides the upregulation of the POR enzyme, the DELLA proteins also regulate the grana stacking in the chloroplast, increasing it and plants with an increased chloroplast grana stacking were more resistant to oxidative damage than WT (Jiang et al., 2012). These observations might suggest a functional relationship between SnRK1.1/NDF4/DELLA in the protection against photo-oxidative damage, albeit our results do not confirm a physical interaction between these proteins.

Once confirmed the interaction between SnRK1.1 and the DELLA proteins, and its independency of the presence or absence of NDF4, we decide to map the protein regions that might be more important for this interaction. The results obtained suggest that both the N-terminal and C-terminal regions are important for this interaction to occur, but the N-terminal region seems to play a more important role (Fig. 3.2). Since SnRK1.1 is a kinase that phosphorylates Ser/Thr residues, these result might indicate that there are more phosphorylatable /Thr residues in the N-terminal of DELLA proteins than in C-terminal, or that interaction depends on other mechanism apart from the Ser/Thr residues that are presumably phosphorylated.

Motifs close to N-terminal of the DELLA proteins are essential but not sufficient to initiate GA-induced RGL2, GAI, RGA and SLN1 degradation, indicating the importance of motifs in the C-terminal region for the GA-sensitivity (Dill et al., 2004; Hussain et al., 2007). The importance of the N-terminal region is well illustrated by the semidominant dwarf phenotype of rga-Δ17 mutants (mutants in which the first 17 amino acid residues, including the DELLA conserved sequence, have been deleted) (Dill et al., 2001). The rice (*Oryza sativa*) DELLA protein SLR1 is phosphorylated in Ser residues, and this phosphorylation occurs in at least two of the three motifs, DELLA, TVHYNP and poly S/T/V (Itoh et al., 2005). This agreed with the fact that motifs close to N-terminal are more important. But as it is mentioned above, residues of the C-terminal have also some importance and several studies have shown that. In a sequence alignment of DELLA proteins from *Arabidopsis*, barley, rice, wheat and maize, identified six conserved Ser/Thr residues, localized in the motifs close to C-terminal (S441, S542, T271, T319, T411 and T535), that are very important to DELLA proteins stability (Hussain et al., 2005). The first conserved heptad leucine repeat in the DELLAs proteins mediate the interaction with PIF (de Lucas et al., 2008), and Hirano et al. (2010) and mutations in the GRAS domain motifs (VHIID, LHRII, PFYRE and SAW) do not allow the interaction of DELLA proteins with GID1, or GID2, or both (Hirano et al., 2010). This suggests that C-terminal motifs are the main responsible motifs for the interaction of DELLA proteins with proteins or gene promoters (Hirano et al., 2010). Hirano et al (2012) also demonstrated that GRAS domain motifs are involved, direct or indirectly, in interaction with promoter region of the target genes or DNA sequences (Hirano et al., 2012). These results agree with the fact that SnRK1.1 have also interacted with the GRAS domain motifs.

Protein phosphorylation/dephosphorylation is a common mechanism involved in signal transduction. The phosphorylated sites can either be directly recognized by interacting partners or introduce allosteric changes that trigger a series of downstream effects (Sun et al., 2012). The phosphorylation occurs in specific regions, with much higher frequency in disordered regions than ordered regions, indicating a strong preference for phosphorylation sites in the intrinsically disordered region (IDR). This may be because the open structure of IDRs reduces the obstruction to access by kinases and phosphatases (Iakoucheva et al., 2004). In a model of disordered protein complexes, phosphorylation has shown to be efficient in the adjustment of the electrostatic interactions of disordered proteins regions for signal transduction (Mittag et al., 2010). The majority of the GRAS proteins have intrinsically disordered region (IDR), and DELLA proteins are included in this majority. The stretches of serine and threonine residues in their intrinsically disordered N-terminal motifs, imply that phosphorylation and dephosphorylation would be a high-probability in these regions. This is consistent with the phosphorylation site predictions of DELLA proteins by combining the localization of the Ser/Thr residues and the disordered structure (Sun et al., 2010; Sun et al., 2011). An increasing number of reports suggests that the DELLA are phosphorylated, but the role of this modification in the control of DELLA activity is unclear. The first observation of DELLA phosphorylation and its effect on DELLA stability suggested that phosphorylation contributed to its degradation (Sasaki et al., 2003). However, increasing evidence supports an opposite effect. Inhibitors of Ser/Thr kinases in SLN1 and RGL2 failed to block the degradation of DELLA proteins (Fu et al., 2002; Hussain et al., 2005). In rice EL1 interact, phosphorylate and stabilized the DELLA protein SLN1. EL1 (earlier flowering1) is a regulator of flowering time in rice (Dai and Xue, 2010). This gene encodes a casein kinase I (CKI), a Ser/Thr protein kinase, which is associated with negative regulation of GA signaling in rice (Kwon et al., 2015). A possible explanations for the first opposite role suggested might be related to the observation that Tyr kinase inhibitors but not phospho-Tyr phosphatase inhibitors block GA-induced degradation of RGL2 protein (Hussain et al., 2007). This results might suggest that phosphorylation of specific Tyr residues would contribute to the DELLA degradation while the phosphorylation of specific Ser/Thr residues would have the opposite effect.

The Y2H and pull down results demonstrated a clear interaction between SnRK1.1 and DELLA proteins. Interestingly, all the DELLA protein sequences present 3 locations (4 in the case of GAI) corresponding to the consensus sequence for phosphorylation by SnRK1 ([hydrophobic]X[R/K]XX[S/T]XXX[hydrophobic] (Vlad et al., 2008)). These results might suggest that SnRK1 is an upstream kinase of the DELLA proteins. A first insight into the physiological role of this interaction was achieved by the phenotypic analysis of SnRK1.1 overexpression mutants in response to GA_3.

In the germination tests, in the presence of PAC the overexpression of SnRK1.1 showed a clear effect of inhibition in the germination ability of seeds. The *rgl2-1* loss-of-function mutant present an almost normal germination (close to 100%) in the presence of PAC (Lee et al., 2002). In the absence of GA_3 *rgl2-1* and *ga1-3 rgl2-1* mutants also shown normal germination. However, the *ga1-3* mutant (that is affected in the biosynthesis of GA, but not in the DELLA) in the absence of exogenous GA_3, shown a very low percentage of germinated seeds, probably due to the stabilization of the DELLA proteins resulting from the total absence of GA_3, (Lee et al., 2002). The *gid1* triple mutant seeds (*gid1a*, *gid1b* and *gid1c*) in even in the presence of gibberellins display a complete suppression of GA responses, failing to germinate, due to the fully stabilization of DELLA proteins (Willige et al., 2007). Stamm et al. (2012) demonstrated that the double mutant *ga1-3 rga-t2* fail to germinate, comparatively to triple mutant *ga1-3 rga-t2 rgl2-1*. This may be due to the stabilized RGL2 of the double mutant, which when mutated in triple mutant, the germination was restored to similar WT levels of germination. In PAC and ABA conditions, this triple mutant demonstrated germination levels similar to WT (Stamm et al., 2012). The hypersensitive to PAC phenotype of the SnRK1.1 OX mutant is similar to the phenotype presented by DELLA loss of function mutants, particularly of RGL2 loss of function mutants, suggesting that SnRK1.1 contributes to a repression of the GA signaling possibly by RGL2 and/or other DELLA protein stabilization.

The plant stem elongation and the hypocotyl growth are process that are positively regulated by gibberellin, and mutants with reduced sensitivity to GA present a characteristic dwarf phenotype. The results obtained shown that the overexpression of SnRK1.1 leads to a reduced effect of GA_3 in promoting hypocotyl growth (Fig. 3.6). In tobacco the overexpression of GAI has a negative effects in the hypocotyl growth, conferring severe dwarfism (Hynes et al., 2003) and a similar results is observed in *Arabidopsis* through the overexpression of RGA and GAI (Feng et al., 2008; Wen et al., 2012). The *35S:rgaΔ17* and *35S:gaiΔ17 Arabidopsis* mutants exhibited a shorter hypocotyl than WT, which cannot be rescued with GA treatment (Feng et al., 2008). Similarly, Wen et al. (2012) demonstrated that the overexpression of GAI protein resulted in reduced GA response compared to control. In absence of GA_3 condition, mutant plants showed 26% reduction in hypocotyl length. In presence of GA_3, control grown 136%, and in the mutant plants GAI overexpression compromised hypocotyl growth, it were 27% longer than untreated seedling (Wen et al., 2012). In these reports, the deletion of the 17aa in the DELLA motif prevents DELLA degradation. The DELLA overexpression mimics the phenotype of *ga1-3* mutants where DELLA proteins are not degraded, which is equivalent to stable proteins. The insensitivity to GA_3 presented by SnRK1 OX plants suggests that SnRK1 is a negative regulator of Gibberellin signaling pathway.

The ability and the timing to flowering are also regulated by gibberellins, which implicates that DELLA proteins are involved in this process. The *Arabidopsis* plants overexpressing SnRK1.1 bloom later than WT. This result evidentiate once again an inhibitory effect of the SnRK1.1 overexpression in the GA-responses. The flowering time of *gid1* triple mutants (*gid1a*, *gid1b* and *gid1c*) is delayed comparatively to the wild type or *ga1-3* mutant plants and the flowers showed structural abnormalities (Griffiths et al., 2006; Willige et al., 2007). Hamama et al. (2012) observed that the overexpression of a DELLA protein of rose (RoDELLA) in Pelargonium effected flowering time. Transgenic plants flowered later and the number of flowering branches was lower than WT, suggesting that overexpression of RoDELLA blocked the rate of floral to vegetative parts (Hamama et al., 2012). Galvão et al. (2012) overexpressed deleted DELLA proteins (deletion of 17 amino acids from the DELLA motif), and the transgenic plants exhibited dark green color and a strong delay in the flowering time. Besides the negative effect of deleted DELLAs in flowering, they conclude that DELLA proteins participate in the regulation of FLOWERING LOCUS T (FT) and TWIN SISTER OF FT (TSF) genes expression (Galvao et al., 2012). The delay in flowering in the presence of GA_3 of SnRK1.1 OX mutants compared to WT show that overexpression of SnRK1 resulted in reduced response to GA.

The phenotypes observed are in agreement with the hypothesis that SnRK1 contributes to the stability of the DELLA proteins. In the last years, several studies have identified the transcription factors (TFs) responsible for the control of those development processes, and demonstrated that these TFs are regulated by DELLA proteins. Among other TFs, the DELLA proteins control the transcription factor SPATULA (SPT) (Gallego-Bartolome et al., 2010; Josse et al., 2011), SCARECROW-LIKE 3 (SCL3) (Zhang et al., 2011), INDETERMINATE DOMAIN1/ENHYDROUS (IDD1/ENY) (Feurtado et al.,

2011) and BOTRYTIS SUSCEPTIBLE1 INTERACTOR (BOI) (Park et al., 2013) that are critical in the regulation of germination; PHYTOCHROME INTERACTING FACTOR (PIFs) (de Lucas et al., 2008; Feng et al., 2008; Gallego-Bartolome et al., 2010; Cheminant et al., 2011; Gallego-Bartolome et al., 2011) and SCL3 (Zhang et al., 2011) that regulate the hypocotyl growth; and SQUAMOSA PROMOTER BINDING–LIKE (SPL) (Yu et al., 2012) and BOIs (Park et al., 2013) that play an important role in the regulation of flowering..

SnRK1 plays a critical role in the regulation of metabolism. Noteworthy, besides their role in the control of developmental processes, the DELLA proteins are also involved in the regulation of some metabolic pathways. In 2006, Cao et al. identify in a screen genes that are DELLA-dependent during germination and flowering. Among these genes, the majority is DELLA–down regulated and a large number encode for enzymes (especially hydrolase, transferase, and oxidoreductase) responsible for the metabolism of carbohydrate, protein, and lipid. This suggested that the metabolic activities are likely kept at a low level (Cao et al., 2006). The observed interaction between SnRK1 and the DELLA might potentiate the necessary coordination in the control of the cells and plant metabolism.

The results obtained clearly shown that SnRK1.1 interact with DELLA proteins and that SnRK1.1 overexpression leads to gibberellin insensitivity. These results are consistent with a model in which SnRK1.1 phosphorylates the DELLA proteins, increasing their stability, and, in this way, represses the response to gibberellin, allowing a global coordinated response to energy stress. When plants are under energetic stress, SnRK1.1 not only control the metabolism of the plant, repressing the anabolism and activating the catabolism, but also phosphorylates and stabilizes the DELLA proteins to repress the developmental processes (germination, hypocotyl growth and flowering) in order to reestablished the energy homeostasis.

FUTURE PERSPECTIVES

5. Future perspectives

The results obtained in this work clearly indicate that SnRK1.1 interact with the DELLA proteins. The preliminary phenotypic analysis strongly suggests that SnRK1.1 in a negative regulator of the gibberellin signaling pathway, likely through phosphorylation of the DELLA protein and its consequent stabilization. However, it would be important to further analyze several aspects of the interaction between SnRK1.1 and the DELLA protein and its physiological relevance.

The interaction between SnRK1 and the DELLA could be confirmed *in vivo* using Bimolecular Fluorescence Complementation assay (BiFC) or co-immunoprecipitation assay.

It would be important to confirm that the DELLA proteins are real substrates of SnRK1 using *in vitro* kinase assays and transient expression assays in protoplast.

A more extensive phenotypic analysis is needed to study de crosstalk between the energy and GA signaling pathway. It would be worth to further confirm the results obtained and to analyze the response to GA of SnRK1 loss of function mutants.

It would also be interesting to study if NDF4 has a role in the interaction between SnRK1 and the DELLA.

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