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Genetic basis of ABA-GA hormonal cross-talk in the control of flowering time in *Arabidopsis*

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Dissertação de Mestrado
Mestrado em Biologia Molecular, Biotecnologia
e Bioempreendedorismo em Plantas

Trabalho realizado sob orientação do
Professor Doutor Alberto Dias
e do
Professor Doutor Lucio Conti

outubro de 2017

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ABSTRACT

Abscisic acid (ABA) and Gibberellins (GA) are two key plant hormones mediating environmental responses. Both hormones play an important role in plant growth and development. Mutants deficient in the production of ABA and GA share phenotypic similarities such as a dwarf phenotype, the production of dark green leafs, and a late flowering phenotype, which suggests that is possible that both hormones share the same influence in the DELLA degradation process and have similar molecular targets. On the other hand literature suggests that ABA and GA are antagonist, for example Gibberellins promotes germination and ABA inhibits it. ABA is commonly related to osmotic/drought stress and the regulation of water balance, but how these effects are integrated with GA is still poorly understood.

GA signaling is mediated by DELLA proteins, belonging to a plant specific family of putative transcription regulators, that act as repressors of GA responses. The current model of GA action proposes that DELLA proteins restrain plant growth whereas the GA promotes growth by overcoming DELLA-mediated growth restraint. To understand the basis of the hormonal cross-talk between ABA and GA, mutants of Arabidopsis DELLA genes – GAI, RGA, RGL1, RGL2 and RGL3 – and mutants involved in ABA biosynthetic and signaling pathways – ABA1 and ABI1 – were crossed and verified by PCR.

In parallel, Western Blot essays were preformed to study the accumulation of DELLA proteins in ABA deficient and signaling mutants. Related to this cross-talk, previous literature shows that there is an accumulation of DELLA proteins in ABA-defective mutants – ABA1. The present study suggests that this accumulation also happens in ABI-defective mutants – ABI1.

Therefore, this supports the idea that ABA has an important role in DELLA function, perhaps through the control of its degradation.

KEYWORDS

ABI1, RGA, GAI, DELLA, hormones

RESUMO

O ácido abscísico (ABA) e Giberelinas (GA) são duas importantes hormonas presentes em plantas que atuam dando respostas em situações de stress. As duas hormonas desempenham um papel importante no crescimento e no desenvolvimento das plantas, também partilham semelhanças fenotípicas como serem anãs, folhas verdes escuras e ambas florescem tardiamente. A literatura sugere que o ácido abscísico e as giberelinas são antagonistas, por exemplo, enquanto que as GA promovem a germinação o ABA inibe-a. O ácido abscísico está diretamente relacionado com a regulação do stresse osmótico e equilíbrio hídrico, porém aparentemente, não existe nenhuma referencia na interação com as proteínas DELLA.

As proteínas DELLA, pertencem a uma família específica de reguladores da transcrição e são repressores intercelulares das respostas mediadas por GA.

O modelo atual da ação GA degrada as proteínas DELLA, que restringem o crescimento da planta, enquanto que a sinalização GA promove o crescimento ao superar a restrição do crescimento mediada pelas DELLA. Estudos sugerem que existe uma relação hormonal entre o ácido abscísico e as giberelinas. Para entender esta relação hormonal, mutantes de Arabidopsis envolvidos na sinalização GA - GAI, RGA, RGL1, RGL2 e RGL3 - e os mutantes envolvidos nas vias biossintéticas e de sinalização da ABA - ABA1 e ABI1 - foram cruzados e testados geneticamente por PCR.

Western Blots foram realizados de modo a entender acumulação das proteínas DELLA nos mutantes - ABA1, ABI1 e RGA.

Simultaneamente, foram estudados múltiplos mutantes envolvidos na sinalização GA e cruzados com mutantes ABA, de modo a eliminar todas as proteínas DELLA e compreender o papel de ABA na degradação das DELLA.

Relativamente à existente relação hormonal entre ABA-GA, a literatura sugere que existe uma acumulação de proteínas DELLA em mutantes ABA nomeadamente, ABA1. O presente estudo indica que esta acumulação também ocorre em mutantes ABI nomeadamente, ABI1.

Ideias que neste caso apoiam a hipótese de que a ABA tem um papel importante na degradação de proteínas DELLA.

PALAVRAS-CHAVE

ABI1, RGA, GAI, DELLA, hormonas

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ABREVIATIONS AND ACRONYMS

ABA – Abscisic acid

ABA1 – Abscisic Acid Deficient Mutant 1

ABA2 – Abscisic Acid Deficient Mutant 2

ABA4 – Abscisic Acid Deficient Mutant 4

ABI1 – Abscisic Acid Insensitive Mutant 1

ABI3 – Abscisic Acid Insensitive Mutant 3

BSA I – Bovine Serum Albumin I – Restriction enzyme

bZIP – Transcriptional Factor

Col – *Columbia*

DELLA – Nuclear Protein, belong to the GRASS family and acts as a growth inhibitor

EB – DNA Extraction Buffer

GA – Gibberellic Acid

GAI – Gibberellic Acid Insensitive Mutant

GID1 – Gibberellin Insensitive Dwarf , GA receptor

LB – Left Border (primer)

LDs- Long days in flowering time

Ler – *Landsberg erecta*

LFY - Floral meristem identity gene

MS – Murashige e Skoog Medium

NCED – nine-cis-epoxycarotenoid dioxygenase

NCO I – Nocardia Corallina - Restriction Enzyme

ORF – Open Reading Frame

PAC – Paclobutrazol

PCR – Polymerase Chain Reaction

PP2C – Protein Phosphatase 2Cs

PYLs – PYR-like Proteins

PYR1 – Pyrabactin Resistance Protein1

RCARs – Regulatory Components of ABA Receptors

Rcf – Relative Centrifugal Force

RGA – Protein

RGL1 – RGA-like 1

RGL2 – RGA-like 2

RGL3 – RGA-like 3

Rpm – Revolutions per minute

SCF – DELLAs degradation complex (SKP1-F-BOX)

SDs – Short days in flowering time

SDS – Sodium Dodecyl Sulphate

SLY1 – SLEEPY1/Gene

SNF1 – Related Protein Kinase

SnRK2s – Related Protein Kinase 2s

SNZ – SNEEZY/ Gene

SOC1 - Transcription activator active in flowering time control

TAE buffer – Tris-Acetato-EDTA buffer

Taq polymerase – *Thermus aquaticus* DNA Polymerase

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1 INTRODUCTION

1.1 The life cycle of *Arabidopsis thaliana*

Under natural conditions, sessile organisms like plants have evolved to adjust their architecture and physiology to overcome environmental challenges (Davière et al., 2016). *Arabidopsis thaliana* is a small flowering plant that belongs to the *Brassicaceae* family, which serves as a model organism to understand complex processes in plants growth and development. The life cycle of *Arabidopsis* includes three consecutive phases including germination, vegetative growth and finally reproductive phase. Germination is the first phase in the life of an *Arabidopsis* plant, where the embryo develops to form a seedling and enters the juvenile vegetative stage. During this phase, the seedling increases its size through a combine process of cell expansion and cell division. Following this phase, the plant attains reproductive competence, being able to flower, thus shifting from the vegetative to reproductive phase. Upon entering the reproductive phase *Arabidopsis* generates an inflorescence bearing to flowers, fruits and seeds. All the phases transitions are regulated according to internal and external cues such as, day length, temperature and hormonal status (Bäurle et al., 2006). *Arabidopsis* is a common model used in research, not only because it is very easy to manipulate genetically but also because of its small genome size which fully sequenced and annotated, the availability of mutant lines and ecotypes already characterized, and its rapid life cycle (5-6 weeks). These rapid cycling plants are known as summer-annual as opposed to the winter-annual *Arabidopsis*, germinating during late summer or autumn and flowering in the following spring after experiencing winter cold.

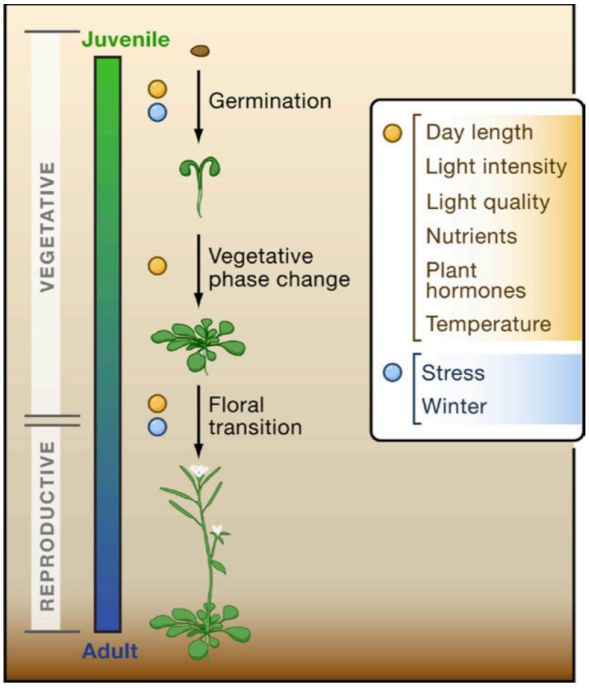


***Figure 1 - Development stages during plants life cycle**. All developmental transitions are regulated by environmental signals such as available nutrients, day length, light intensity, light quality, and ambient temperature as well as endogenous signals transmitted by plant hormones. Cold temperature and stress affect both germination and floral transition. (Bäurle et al., 2006).*

1.2 The floral transition in *Arabidopsis*

The transition to flowering is a major developmental switch in the life cycle of plants, when the shoot apical meristem (SAM) changes from vegetative to reproductive development. After the floral transition the SAM turns into an inflorescence meristem (IM) generating floral meristem (FM), instead of leaves, which gives origin to floral organs. After the flower transition the SAM is irreversibly changed to a IM (Liu et al., 2010). There are four main pathways that participate in floral transition including the photoperiod pathway, the vernalization pathway, the autonomous pathway and the gibberellin pathway.

The photoperiodic pathway, is one of the main regulatory components of the floral transition, which allows to classify plants into two groups : long-day plants (LDPs) and short-day plants (SDPs) (Garner et al., 1922). *Arabidopsis* is a facultative long-day plant that flowers earlier under long- day conditions (LDs, 16 h of light typical of temperate summer days) than under short-day conditions (SDs, 8 h of light, a typical winter day) (Gregory et al., 1953). The vernalization response consists of a cold temperature treatment that induces/accelerates flowering. It ensures that flowering only happens during spring when the environmental conditions are favorable for growth and development of the plants (FCA, a Gene Controlling Flowering Time in *Arabidopsis*, Encodes a Protein Containing RNA-Binding Domains).

In *Arabidopsis*, the phytohormone Gibberellic acid (GAs) is required for flowering under SDs, when the inductive photoperiodic pathway is not active. Under SDs, bioactive GAs promote the floral transition. Meanwhile under LDs the role of GAs in promoting flowering is less pronounced but still present (Blazquez et al., 1998; Moon et al., 2003; Porri et al., 2012).

Besides the four major flowering pathways, the floral transition is strongly influenced by temperature, age of the plant and drought stress related components. Abscisic acid (ABA) acts as a endogenous messenger in response to drought stress, as drought triggers ABA accumulation in plants (Verslues et al., 2011; Zeller et al., 2009).

1.3 Phytohormones

There are a variety of plant hormones that regulate growth and development, among them there are ABA and GA. The role of ABA and GA appears to be tightly related to responses to abiotic and biotic stress. Although ABA and GA responses are relayed through distinct signaling pathways several studies suggest the existence a complex network of interaction and feedback regulations between the two hormones (Anderson et al. 2004 ; Fahad et al. 2015).

1.3.1 Abscisic acid (ABA)

The plant hormone abscisic acid (ABA) plays a major role in seed maturation and germination, as well as in adaptation to abiotic environmental stresses. Other ABA actions involve modifications of gene expression, and the analysis of ABA responsive promoters has revealed a diversity of potential cis-acting regulatory elements (Leung et al, 1998).

Drought, salinity and a low temperature are some common environmental factors which plants must overcome. Plants respond to these adverse conditions commonly through adapting their physiology and morphology to sustain growth (Lovell et al., 2015; Ludlow et al., 1989). Under non-stressful conditions, ABA in plant cells is maintained at low levels, but some low levels of ABA are required for normal plant growth (Xiong et al., 2003). ABA, acts as an endogenous messenger in response to drought stress, it was showed that drought triggers ABA accumulation in plants that consequently induces stomatal closure and enhancers the expression of stress related genes (Verslues et al. 2011; Zeller et al. 2009).

Abscisic acid Biosynthesis

ABA is a plant phytohormone whose biosynthesis starts in the plastids by cleavage of C40 carotenoids. The first step of this biosynthesis is the transformation of zeaxanthin into violaxanthin catalyzed by the zeaxanthun epoxidade ABA1. Next the violaxanthin is converted into xanthoxin through the action of ABA4 (an intramolecular oxiredutase) and nine-cis-eposycarotenoid dioxygenase (NCED) enzymes. Xanthoxin is presumed to migrate to the cytosol by an unknown mechanism, because the following reactions takes place in the cytosol. The last two steps are catalyzed consecutively by ABA2, which converts xanthoxin to abscisic aldehyde, and ABA3 for the last oxidation of the abscisic aldehyde to abscisic acid (ABA) as illustrated in figure 2 (Seo et al., 2011).

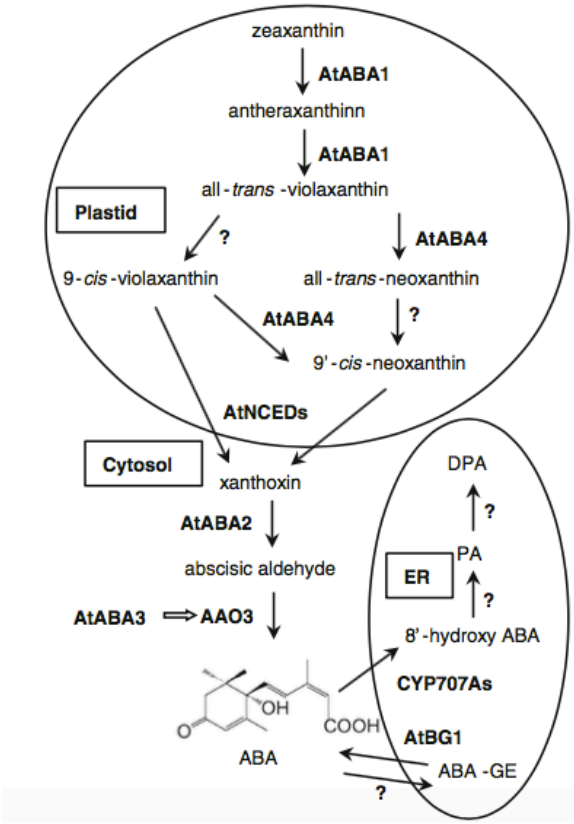


Figure 2 - ABA biosynthetic pathway. *Pathway is summarized indicating all metabolic intermediates from zeaxanthin to ABA, the enzymes responsible of each step and the subcellular compartment where ABA intermediates are produced. Unidentified biosynthetic steps are indicated with a question mark "?".*

Abscisic acid Signaling

The main role of ABA apart from its functions in plant growth and development, is to regulate water balance and osmotic stress, and for that ABA signaling is important for the plant performance under conditions of limiting water availability. Genetic screens in *Arabidopsis thaliana* identified many downstream ABA signaling components. Recent findings in the field of ABA signaling reveal a unique hormone perception mechanism (figure 3) where ABA binds to the ABA receptors (Fernando et al., 2016).

Three proteins classes compose the core ABA signaling, the ABA receptor – known as REGULATORY COMPONENTS OF ABA RECEPTOR (RCAR), or PYRABACTIN RESISTANCE PROTEIN1 (PYR1) and PYR-like Proteins (PYLs) –, the PROTEIN PHOSPHATASE 2Cs (PP2Cs), that act as negative regulators of the signaling process and SNF1-RELATED PROTEIN KINASE 2s (SnRK2s) that act as positive regulators downstream of the signaling process. The recently identified ABA receptors can be found in cytoplasm as well as in the nucleus , as illustrated in figure 3 (Guo et al., 2011).

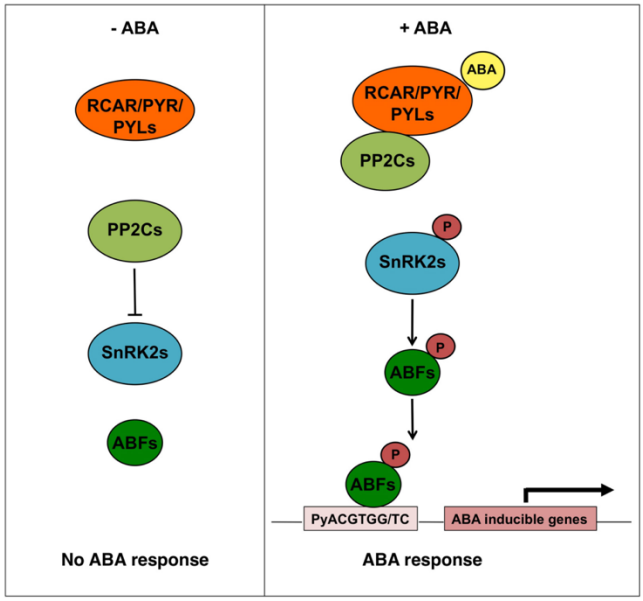


Figure 3 - ABA signaling pathway *– illustrated pathway shows summarized interactions between receptors PYR/RCAR in the presence and absence of ABA and ABA mediated response. (Fernando et al., 2016).*

Upon perception of a stress signal, ABA is induced primarily in the vascular tissue in *Arabidopsis* (Endo et al., 2008; Koiwai et al., 2004). In presence of ABA, ABA binds to the PYR/RCAR ABA receptors and this complex inhibits the action of group A PP2C proteins allowing the SnRK2s activation and phosphorylation of their targets. In the absence of ABA, PP2Cs dephosphorylate SnRK2s inhibiting their kinase activity, thereby preventing downstream gene expression. The genetic suppression of PP2Cs activities allows the activation of SnRK2s and constitutive ABA signaling (Nishimura et al., 2010; Raghavendra et al., 2010).

PYR/PYR1-LIKE (PYL)/RCARs are soluble proteins that directly bind through non-polar/ hydrogen bonds to ABA. In the absence of ABA, PYR/PYR1-LIKE (PYL)/RCARs produce protein dimers, but if ABA binds to them, their conformational structure changes into a weaker homodimer structure (Nishimura et al., 2009; Santiago et al., 2009). These conformational changes allow the catalytic interaction between PYR/PYR1-LIKE (PYL)/RCARs and PP2C proteins. However, in the presence of ABA this interaction does not happen and the PP2C proteins are inhibited (Takeuchi et al., 2014).

On the other hand, SnRK2s are also involved in the phosphorylation of some bZIP transcriptional factors (Johnson et al., 2002). Other transcriptional factors that are not influenced by the SnRK2 phosphorylation play a major role in ABA response such as ABA INSENSITIVE 3 (ABI3), a B1/B3 transcription factor responsible for the positive and negative regulation of ABA responses. ABI3 protein presents four different domains A1, B1, B2 and B3 starting from the N-terminal, and has a significant function in development that

includes controlling flowering time (Verslues et al., 2011; Zhang et al., 2006).

Abscisic acid insensitive 1 (abi1)

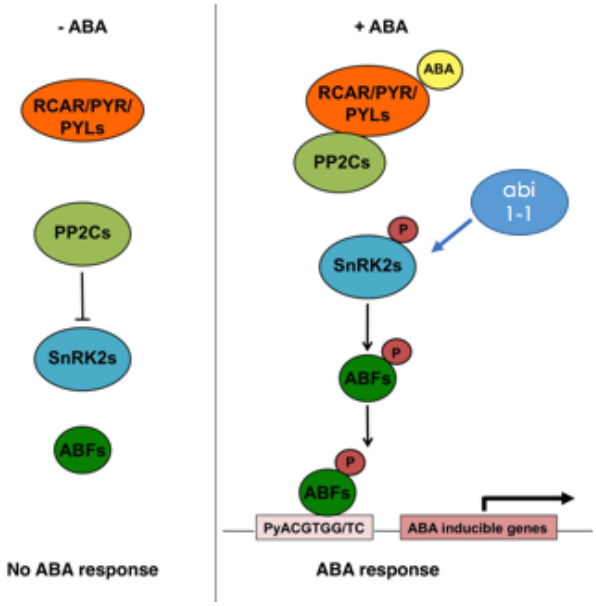


Figure 4 - ABA signaling pathway *- In the presence of a constitutive mutation abi 1-1 that belongs to the family of protein phosphatases PP2Cs, that is required for the kinase SnRK2 activation and consequent ABA mediated response. (Belin et al., 2006; Vlad et al., 2009).*

ABA insensitive mutants allowed to better understand the mode of ABA signaling. In this project, we use a specific mutant line named *abscisic acid insensitive mutant 1-1* (*abi1-1*). Arabidopsis *abi1–1* mutant have a reduced seed dormancy, enhanced water loss, and a decreased ABA sensitivity of stomatal regulation. The abi1-1 protein is a constitutively active version of PP2C, which derives from an amino acid substitution Gly to Asp at position 180. The mutation affects an early step in the ABA signal transduction pathway. Yeast two hybrid assays revealed that the abi1-1 protein is insensitive to the PYR/RCAR mediated binding. Therefore the abi1-1 mutations confers a constitutive binding to the SnRK2s, thus blocking the signal cascade. However, it should be pointed out that this gain-of-function allele produces a mutant proteins lacking protein phosphatase activity. Given the dominant nature of *abi1-1* mutation, it is plausible that blockage of ABA signaling is because of a protein-mediated sequestration effect rather than a reduction in phosphatase activity (Yan et al. 2003).

1.3.2 Gibberellic acid (GA)

GA was first identified in the pathogenic fungus *Gibberella fujikuro* as a metabolic product, similar in physiological properties to the gibberellins described by Japanese investigators (Brian et al., 1954). They are another plant phytohormone which influences plant development processes including germination, stem maturation, leaf expansion, trichrome

development, pollen maturation and the induction of flowering (Achard et al., 2009). GA is well known to play an important role in regulating the timing of the floral transition. GA-deficient mutants are dwarfed and late- flowering, and treatment of these plants with GA restores normal growth and flowering time.

The gibberellin signaling pathway in Arabidopsis, is regulated by 5 DELLA proteins named GAI, RGA, RGL1, RGL2 and RGL3. All of them act as negative regulators of GA signaling.

DELLA are part of the GRAS domain family protein, which are a family of putative transcription regulators, involved in several different aspects of plant growth and development in addition to GA signaling. The GAI gene were originally defined by the cloning of the dominant mutant GA insensitive (*gai*) allele (Peng et al., 1997). gai encodes a mutant DELLA protein that lacks a region of 17 amino acids at its N-terminus (which includes the amino acid string D E L L A) and confers a dominant dwarf, reduced GA-response phenotype. The role of RGL2 is to prevent germination after imbibition and GA promotes germination by down-regulating RGL2 accumulation. GA regulates stem elongation via GAI and RGA, and seed germination via RGL2. RGL2 acts as an integrating factor that links GA signaling and environmental cues in the regulation of seed germination. (Gibberellin regulates Arabidopsis seed germination via RGL2, a GAI/RGA-like gene whose expression is up-regulated following imbibition) (Lee et al., 2002).

Gibberellin Signaling

In the past studies the components of GA signaling were identified in *Oryza sativa* and *Arabidopsis thaliana*. The key components include GID1(GIBBERELLIN INSENSITIVE DWARF1) which acts as GA receptor, the DELLA growth inhibitors (DELLAs) and the F-box proteins SLEEPY1 (SLY1) and SNEEZY (SNZ) in *Arabidopsis* (Achard et al. 2009). The current model of GA action proposes that DELLA proteins restrain plant growth whereas the GA signal promotes growth by inducing DELLA proteins degradation. Mutants with constitutively active GA responses have taller stems, paler green leaves and lower fertility than wild-type plants, irrespective of bioactive GA content. Genetic analysis of the GA-response mutant categories led to the current model that GA acts as an ‘inhibitor of an inhibitor’ (Harberd et al., 2009). Supporting the importance of DELLA degradation, the original gai dominant allele generates a DELLA protein that is relatively resistant to the effects of GA and, therefore, maintains repression irrespective of the presence of GA.

1.4 DELLA Proteins

DELLA proteins are distinguished from the rest of the GRAS family by a specific N-terminal sequence containing two conserved domains: the DELLA domain and the TVHYNP domain. Studies reveal that GA is also responsible for the degradation of these proteins by using a complex called the 26S-proteossome. Mutant analysis has revealed that GRAS proteins regulate several different aspects of plant growth and development in addition to GA signaling. (McGinnis et al., 2003) On one hand, in the absence of GA there is an accumulation of DELLA that consequently represses GA mediated responses. In the presence of GA, GA binds to its receptor GID1 to create the GA-GID1-DELLA complex. This complex enhances the affinity between DELLAs and SCF E3 ubiquitin-ligase complex, which promotes DELLA degradation by the 26S proteasome. Thus, GA promotes growth by mediating the proteasome-dependent destabilization of DELLA proteins (figure 5) (Dill et al., 2004; Lechner et al., 2006; Goomi et al., 2004).

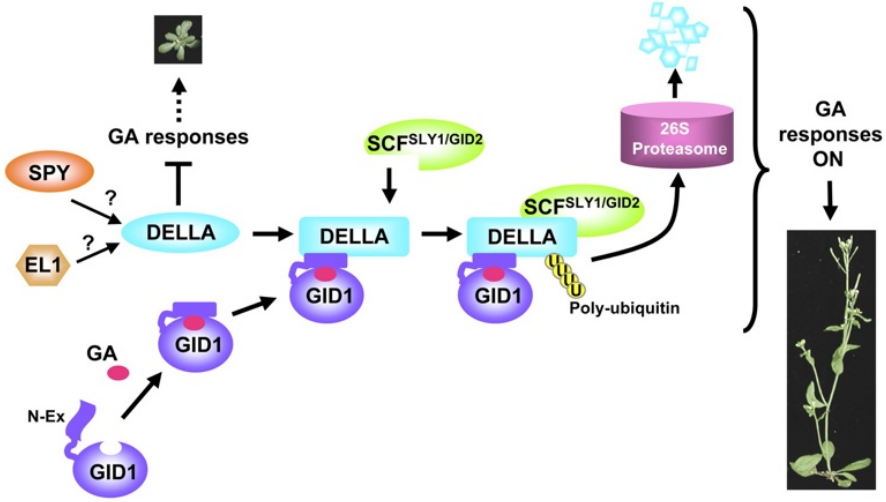


Figure 5 - Process of Della degradation mediated by the 26S-proteossome, and the GA response. *GA binds to its receptor GID1 that stimulates the formation of the GA-GID1-DELLA complex. In the presence of GA, DELLA proteins can be degraded by attaching to the SLY1 F-box (in Arabidopsis) and later guided the 26S proteasome to suffer degradation thereby relieving their growth restraining effects (Sun et al., 2010).*

In Arabidopsis, the DELLA-GA system regulates floral initiation via transcripts of the floral meristem identity genes *LEAFY* (*LFY*) and *SUPPRESSOR OF OVEREXPRESSION CONSTANTS 1* (*SOC1*). DELLA delays flowering in short-day photoperiods (SDs) by repressing the up-regulation of LFY and SOC1 transcripts. (Achard et al., 2007)

1.5 Cross talk between hormonal pathways

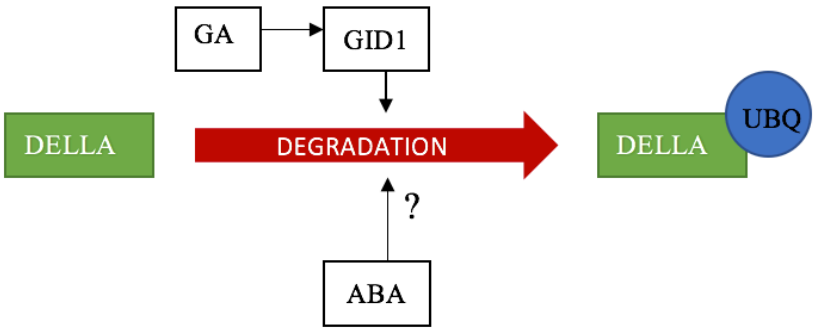


Figure 6 *– Brief illustration of DELLA degradation, suggesting a hormonal cross-talk between hormones ABA and GA and the role they both play in DELLA degradation. The interaction of ABA marked with "?" is not known yet.*

Recent studies indicate that the signaling outcome of individual hormonal effects could be rather the result of different hormonal pathways that are interconnected through a network of interactions and feedback regulations (Kuppusamy et al., 2009; Vanstraelen et al., 2012). The physiological relevance of such hormonal cross-talks may be that to confer plasticity to plant development, since individual hormones often convey specific information. GA, through its role in the regulation of DELLA, plays a key integrative role.

The literature suggests that ABA and GA are normally antagonistic. For example, GA promotes germination whereas ABA inhibits it (Piskurewicz et al., 2008). After germination, the mode of cross talk between GA and ABA is much less clear.

ABA and GA are two key plant hormones mediating environmental responses. The main role of ABA apart from its functions in plant growth and development, is to regulate water balance and osmotic stress, and for that ABA signaling is important for the plant performance to overcome these problems (Fernando et al., 2016).

Therefore, while in most cases ABA and GA are antagonist there are instances where they can act in synergy (Lee et al., 2002).

In this project, molecular approaches were preformed to create mutants involved in GA signaling and ABA biosynthetic and signaling pathways. The aim of this study is to provide insights into the potential ABA-GA cross-talk and its role in the degradation of DELLA proteins.

2 MATERIALS AND METHODS

2.1 Plant Material

In this study, we analyzed wild type and mutants of *Arabidopsis thaliana* both in ecotype *Columbia* (Col-0) and *Landsberg erecta* (Ler-0).

Table 1 - *Genotypes and respective sources used in this study, mechanism used to research was Tair website (https://www.arabidopsis.org/).*

Allele	*Background*	*Reference*
abi1-1	*L.er.*	*Verslues, et al. 2006*
aba1-6	*Col-0*	*Niyogi et al., 1998*
gai-100	*Col-0*	*SAIL_587_C02*
rga-100	*Col-0*	*Marín-de la Rosa, et al. 2015*
rgl1	*Col-0*	*Tyler, L. et al. 2004*
rgl2	*Col-0*	*SAIL_345_F05*
rgl3	*Col-0*	*SAIL_349_B10*

2.1.1 Plant growth condition in vitro

Seeds were sterilized before sowing on plates for in vitro growth. For sterilization, seeds were placed in 1,5 mL Eppendorf tubes in the presence of 500 µl of sterilizing solution - 70% v/v ethanol (EtOH), 0.1 % (w/v) Sodium Dodecyl Sulphate (SDS) -. Two washes were done for 5 minutes, each one with agitation. After the two washes the solution was removed with a pipette and replaced with ethanol 100% (v/v) for another two washes of 5 minutes in agitation.

Seeds were transferred to a sterile filter paper and air-dried under a laminar flow hood before sowing onto Petri dishes filled with approximately 25 mL of Murashige e Skoog (MS)-Agar growth medium. The MS medium was prepared by dissolving 4.3 g of ready-made MS salt mix and 5 g of Sucrose in two liters of distilled water. The solution was adjusted to pH 5.8/5.9, and 2.8 g of Plant agar was added for 500 ml? before autoclaving. Sterilized seeds were distributed onto solidified MS-Agar plates. Plates were first placed at 4 °C for stratification and finally moved into a growth chamber set with a mean temperature of 22 °C and continuous light.

When appropriate, mutants were selected with BASTA, a non-volatile herbicide with non-selective activity against many broadleaf weeds and grasses, or kanamycin that is an antibiotic. Both were added to the MS in a proportion of 1:20000, that means that 25 µl of

stock BASTA solution (X mg/ml) in 50 mL of MS, or 1:1000, that means 50 µl of (y mg/ml) in 50 mL of MS for kanamycin.
This method allows to see the segregation between the homozygous and heterozygous plants in each background and only transferring the resistant ones into the soil.

2.1.2 Plant growth condition on soil

Seeds were sown directly on soil. Seeds were sowed and plants grown in a controlled environment, at a temperature of 20 ºC to 23 °C, 55 % relative humidity, under long day (16 h light / 8 h dark) conditions. Light was provided by fluorescent tubes at a fluency of approximately 80 micro Einstein. The soil used was a 1:2 mix of peat and sandy soil.

2.1.3 Crossing

Arabidopsis thaliana is a hermaphrodite, it tends to self-pollinate, so different genotypes were created and cross pollination was performed. All crosses are illustrated in the following scheme:

Crosses	aba-1-1 *pRGA:RGA:GFP* = *Available*
	abi 1-1 x *pRGA:RGA:GFP*
	rgl1 x *rga-100 gai-100*
	rgl2 x *rga-100 gai-100*
	rgl3 x *rga-100 gai-100*
	rga-100 gai-100 x *aba 1-6*
	rgl3 x *aba 1-6*

Crosses were done by opening the flower before it was ready to bloom, and the stamens were pinched off with tweezers while leaving the carpel intact. Pollen was obtained from a donor plant: a flower was taken and placed on the top of an emasculated flower, to make the pollen fall on the stigma of the carpel. Successfully-pollinated flowers developed siliques. The seeds (F1 generation) were harvested once the siliques were dry. F1 plants were typically genotyped by PCR with molecular markers, to confirm that the crosses succeeded.

2.2 Molecular Methods

Before starting the DNA extraction, samples were collected from young leaves, because they yield higher quality DNA compared to older ones. In order to obtain better results, the PCR

was preformed right after the extraction.

2.2.1 DNA extraction

Plant tissues were placed in 1.5 mL Eppendorf tubes and immediately homogenized with a pestle with 300/400 µl of DNA extraction buffer (EB) (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% w/v SDS). The homogenized tissues were clarified by centrifuging at 15000 rpm for 15 minutes at room temperature. Then the supernatant was removed to new Eppendorf tubes already filled with 300/400 µl of IPA 100% (v/v) to precipitate nucleic acids.

After a quick vortex, the supernatant was kept at 10 ºC for 10 minutes before centrifugation at 15000 rpm for another 10 minutes. The DNA was pelleted by centrifugation at 15000 rpm for 5 minutes at room temperature. The supernatants were discarded and pellets washed with 500 µl of 70% (v/v) ethanol.

After the ethanol was discarded, the tubes were dry in a sterilized paper until the ethanol we completely out of the tube. Finally the pellet was re-suspended in 20 µl of TE buffer (10mM Tris pH 7.9, 1mM EDTA), and storage at -20 ºC

2.2.2 PCR

Mutant genotypes were identified by PCR (Polymerase Chain Reaction) amplification. To identify a T-DNA insertion mutant, a primer couple flanking the insertion site was used. With these primers pair a PCR reaction amplifies a specific region in the wild type, but not in a T-DNA insertion mutant plant (due to the presence of the T-DNA between the flanking primers). T-DNA insertions were confirmed by using a primer specific for the left border (LB) of the T-DNA.

To genotype point mutations, PCR fragments were subject to a digestion with a specific restriction enzyme (BSA I / NCO I) at 37ºC for at least 2h/3h to distinguish mutant and wild type alleles.

The general PCR reaction conditions were:

94 °C	1 min	
94 °C	**15 sec**	**X 35 cycles**
T °C	**25 sec**	
72 °C	**Time**	
72 °C	5 min	
15 °C	∞	

Table 2 - *Primers used to preform genotyping in all the genes. Most of the primers where already available, others needed to be designed and ordered.*

TARGET GENE	FORWARD PRIMER	REVERSE PRIMER
ACTIN	CTCTCCCGCTATGTATGTCGCCA	GTGAGACACACCATCACCAG
abi1-1	ATCCGTAAGGTACCATGGAGGAAGTATCTCCGGCG	ATCCGTAACTCGAGCCGTTCAAGGGTTTGCTCTTGAG
aba1-6	GCTCGGAGTAAAGGCGGCGA	CAGGAAGTCCCCGTGACGCC
gai-100	CAACCATGAAGAGAGATCATCATC	CTTGGAAACTCTCCACCAATTAG
rgl1	CATCAATGACGACGGT	TTATTCCACACGATTGATTCGCCA
RGL2	CACCATGAAGAGAGGATACGGAG	GGCGAGTTTCCACGCCGAGG
rgl2-1	GAGCTCCACCGGTTATTAGCCC	SAIL_345_F05
RLG3	CTGGCTCAAGCCATCGGCGTC	AATGAAAACCTCTCAAAAAACCC
rgl3-1	AATGAAAACCTCTCAAAAAACCC	TGGTTCACGTAGTGGGCCATCG

The PCR programs were adjusted for the different couple of primers in terms of annealing temperature and time according to the length of the predict PCR product.

Different PCR reactions were set up, depending on the purpose of the experiment and a home-made taq polymerase was used. The consistent mix used was:

- 15.1 µl dH2O MilliQ (deionized water)
- 2 µl Buffer 10X
- 0.4 µl dNTPs 10 mM
- 0.6 µl Forward primer 10 µM
- 0.6 µl Reverse primer 10 µM
- 0.3 µl Homemade Taq polymerase

The final solution used was: 19 µl of PCR mix + 1 µl DNA extract, reaching a total of 20 µl.
In some cases, different taq polymerases were used, such as:

For abi1-1, the GOTaq (NEB) was used:

- 16,05 µl dH2O MilliQ (deionized water)
- 5 µl Buffer 10X
- 0.5 µl dNTPs 10 mM
- 0.6 µl Forward primer 10 µM
- 0.6 µl Reverse primer 10 µM
- 1.25 µl Homemade taq polymerase
- 1 µl DNA template

The final solution was a total of 25 µl.

For genotyping aba1-6 REDtaq was used:

- 18.25 µl dH2O MilliQ (deionized water)
- 2.5 µl Buffer 10X
- 0.4 µl dNTPs 10 mM
- 0.6 µl Forward primer 10 µM
- 0.6 µl Reverse primer 10 µM
- 1.25 µl Homemade Taq polymerase

The final solution was: 24 µl of PCR mix + 1 µl DNA extract reaching a total of 25µl.

2.2.3 Gel electrophoresis

PCR products were size-separated on agarose gels. Agarose gels were made by melting different quantity of agarose powder (SIGMA) in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA) to obtain gels at different concentrations. For example, in a 1% (w/v) gel with 1 g of Agarose was dissolved (in a microwave oven), or a 2% (w/v) gel with 2 g of agarose, when the expected bands were too low, in 100 mL of TAE. 10 mg/ml of Ethidium bromide (EtBr) was then added to the molten gel (1.5 µl per 100 µl of gel). Ethidium bromide is fluorescent intercalating dye that was used to visualize the nucleic acids fragments under a UV trans illuminator. The molten gel was poured into a casting tray fitted with comb-shaped mold. The electrophoresis was carried out by applying current to the running chamber. The gel box was filled with TAE, making sure all the wells were submerged.

The samples were mixed with an appropriate amount of 5X loading buffer – 0.25% Bromophenol Blue, 30% Glycerol – to increase sample density and thus facilitate loading into the wells. The first well of the gel was loaded with a 1 kb DNA ladder, containing linear DNA fragments of known length to estimate the size of the migrated fragments. Finally, the gel was left to run approximately 15 minutes.

2.2.4 Protein extraction and quantification

For protein extraction the Buffer E method was used (Martínez et al., 1999) and a Western Blot could be performed to check the expression of a particular protein. Firstly, the fresh tissue was homogenized in 1.5 mL Eppendorf's with 100 µl volume of Buffer E per 100mg of fresh sample weight. After a brief vortexing, samples were spun at maximum speed in a bench centrifuge at room temperature for 10 minutes. The supernatants were recovered and quantified with a spectrophotometer. 30 µl was stored for further uses under -80 ºC and 2 µl for the quantification.

Protein quantification was done by using the Bradford method (Bradford, 1976) by mixing 2 µl of protein extract, 18 µl dH2O and 780 µl Bradford reagent. The blank controls contained

18 µl dH2O and 2 µl buffer E. Reference protein standards were BSA 100, 200 and 400 ng/ul made by mixing various amounts of stock BSA solution 1 mg/ml, dH2O and 2 µl buffer E in a total volume of 20 µl .

The absorbance of both samples and controls were recorded at λ = 595 nm. The absorbance obtained allowed to calculate the necessary amount of protein that needed to be load on gel. 10 µl of Laemmli loading buffer was added to each sample with 30 µl of proteins extract and the mix was boiled at a heated at 75 ºC for 10 min.

2.2.5 SDS PAGE gel

SDS-PAGE is a technique used to separate proteins according to their molecular mass. SDS, a strong ionic detergent, denatures proteins and confers them a negative charge. Therefore proteins are size fractionated largely according to their molecular weight and not charge. Polyacrylamide consists of bisacrylamide cross-links between two molecules of acrylamide. This polymerization reaction is promoted by ammonium persulfate and TEMED (N,N,N',N'-tetrametiletilendiammina), added to the polyacrilamide solution together with a buffer and SDS. The polyacrylamide gel concentration is adjusted according to the molecular weight of the protein that we want to resolve. An SDS-PAGE gel consists of a stacking gel in which proteins are first packaged to allow the following separation in the running gel.

Table 3 *- Loading amount of protein for the gel calculated in an excel file for a SDS Gel (2x)*

Reagents	Polycrilamide gel 8%	Stacking gel 5%
dH2O(mL)	4.7	2.85
Buffer(mL)	2.5	1.25
30% AC/B/S(µl)	2.7	0.85
SDS 10%(µl)	100	50
APS(µl)	50	25
TEMED(µl)	10	5
Total (mL)	10	5

During the preparation of the gels it was important to avoid the formation of bubbles, since oxygen delays the polymerization. The running gel mixture was poured between two glasses placed in a gel caster and left to polymerize in the presence of an isopropanol layer to flatten the gel top. The 5% stacking gel was prepared and the mixture was gently added over the solidified running gel after removing isopropanol, and a comb was inserted at the top. Once

the gel was polymerized, the electrophoresis apparatus was assembled and filled with the SDS-PAGE running buffer 1X. Running Buffer 10X for 1 L (Tris-base 30.3 g, Glycine 144 g SDS 10 g). Using the data obtained from the quantification, the same amount of proteins from each sample (30 µg ca.) were loaded onto the gel (one sample for each well), together with a pre-stained marker. A constant voltage of 150 Volt was provided for 55 minutes. The grid was placed into a transfer chamber (filled with transfer buffer) and a constant voltage of 100 Volt was applied for an hour.

Proteins were blotted onto PVDF membrane, which was then incubated in a solution of 5 % milk for 1 hour. The primary antibody was incubated over night at 4C. The membrane was washed 4x with TTBS (Buffer TTBS 10X : Tris-HCl pH 7.5/8.0 (BioRad) 200 mM NaCl (Sigma) 1.5 M, Tween-20 (Sigma) 2%) and the incubated again with the secondary antibody labelled with horseradish peroxidase (HRP) for 1 hour. Filters were washed again 4x with TTBS before incubation with the HRP substrate (Millipore) and signals were digitally recorded with a ChemiDoc apparatus.

3 Results and Discussion

3.1 Is ABA involved in the degradation of DELLA proteins?

Previous studies done in the lab have led to suppose that ABA and GA control in a similar fashion different aspects of plant development including growth and flowering time. It is thus hypothesized that they can also converge to the regulation of DELLA proteins.

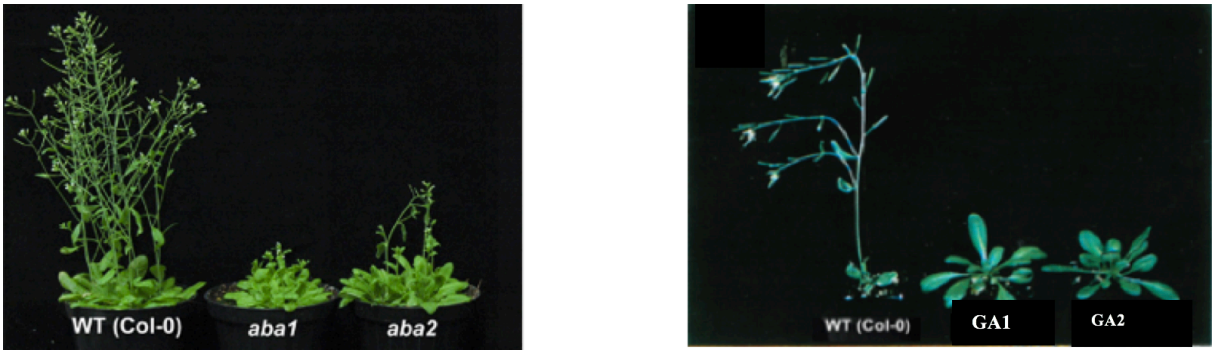


Figure 7 - *By comparing ABA and GA deficient mutants, we can see clearly observe that both hormones are required to accelerate the floral transition. (Riboni, Matteo, et al.2013) (Reeves, Paul H., and George Coupland.200)*

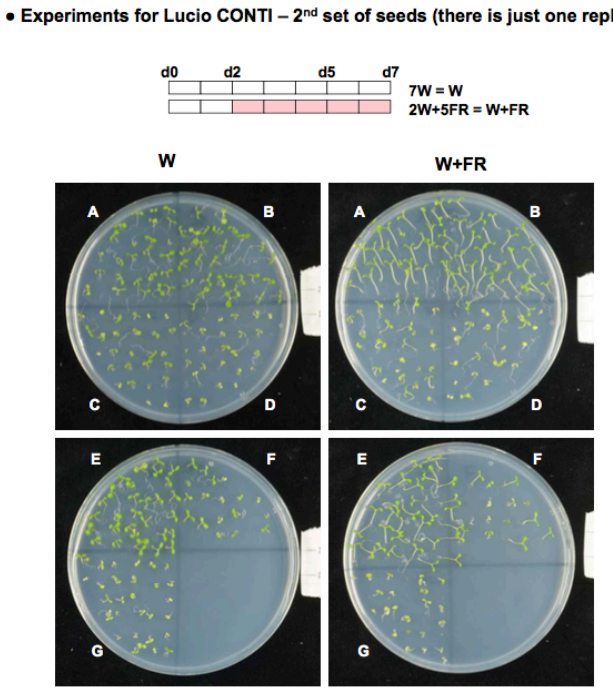


Figure 8 - *Seedlings from (A) Col stock I; (B) Col stock II; (C) aba1-6 stock III; (D) aba1-6 stock IV; (E) Ler; (F) Ler abi1-1 2015; (G) Ler aba1-2 B, were grown on plates with MS and exposed under white (W) and far-red light (W+FR) for a period of seven days.*

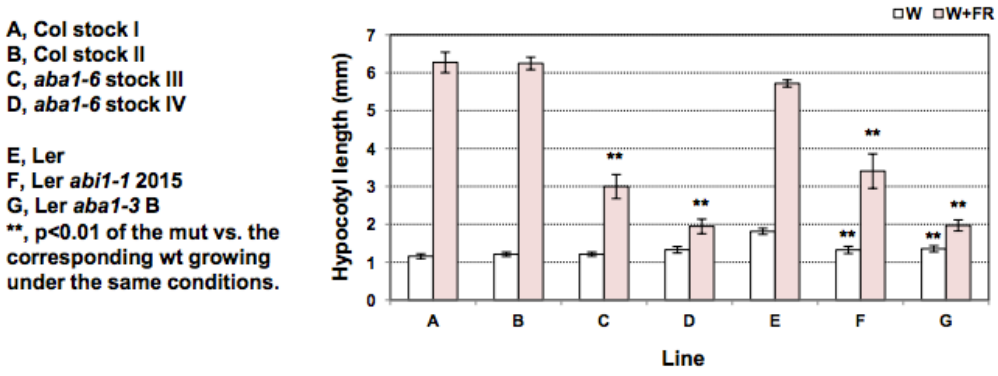
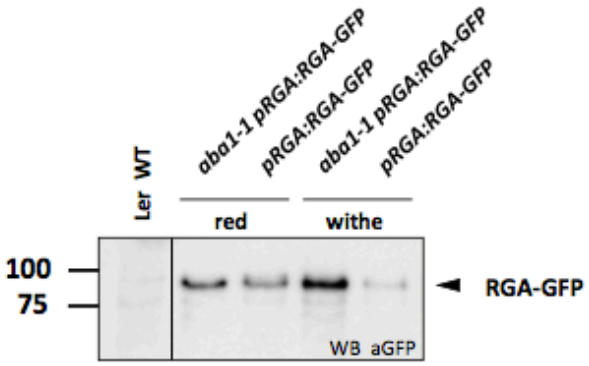


Figure 9 *- Results provided By Martinez Group in collaboration with Lucio Conti Lab, showing elongation of the hypocotyl under far-red light and comparing mutants with the wild type under the same condition.*

One striking example pertains the control of hypocotyl growth under Far Red (FR) irradiation (which stimulates growth in part through GA production). FR had a very mild effect in ABA deficient (*aba1-3* and *aba1-6*) and signaling mutants (*abi1-1*). The data (figure 8 and 9), produced by the Martinez group in Barcelona in collaboration with Professor Lucio suggests that ABA also promotes growth perhaps by affecting GA signaling or production. In line with this idea, studies performed in the lab showed an over accumulation of RGA, one of 5 DELLA proteins, in ABA-deficient mutants (Fig 10 and 11).



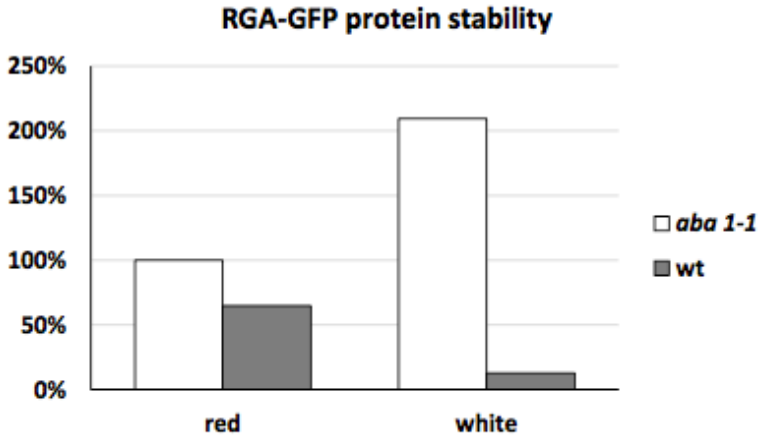


Figure 10 - *Results about RGA protein stability essay in wild type and mutant line.*

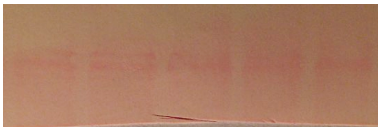


Figure 11 - *Ponceau stanning of the filter confirms that the protein was loaded in the same amount.*

3.2 Detection of DELLA in ABA insensitive mutants

My first goal was to verify the significance of the above protein results independently in an ABA insensitive background. I thus aimed at generating double mutants of Arabidopsis *abi1-1* which contain the transgene *pRGA:RGA-GFP* (to detect variations in RGA levels).

Two segregating population for *abi1-1* but with the transgene *pRGA:RGA-GFP* in an homozygous state (based on absence of segregation on Kanamycin selection) was already available for molecular screening as already described in the material and method section.

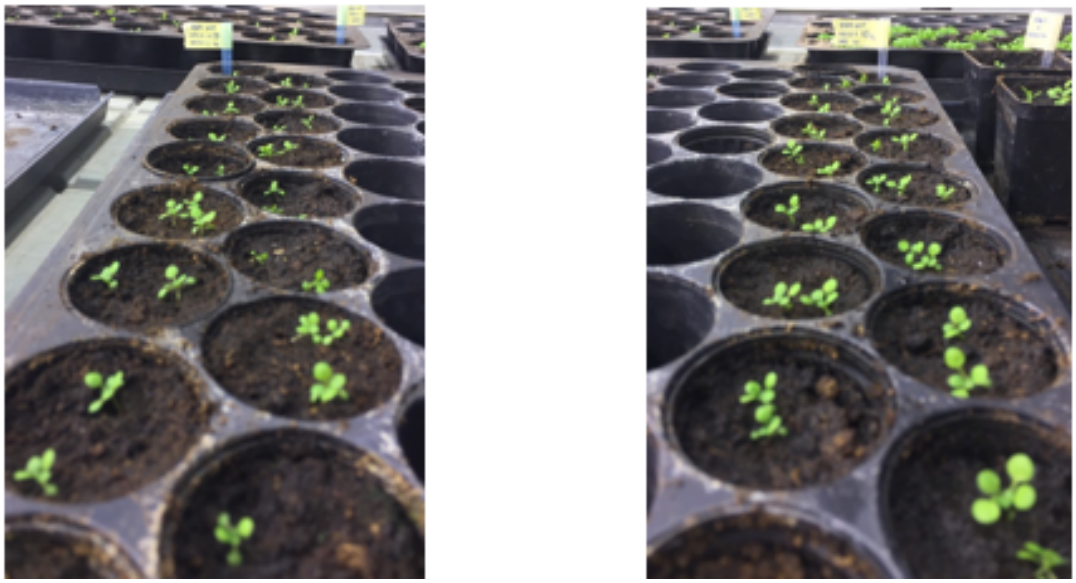


Figure 12 - *Heterozygous plants on the left abi1-1p:RGA:GFP line 15 and on the right line 19, 2 weeks old, growing on soil.*

When the plants were 2 weeks old, a DNA extraction followed by a PCR, were performed to test the homozygosity of the lines 15 and 19 for *abi1-1*. Specific primers, already published, for this gene, lcm48 and mr130 did not produce consistent results. Tests were thus carried out using ACTIN primers to check the quality of my DNA extractions. Because Actin amplification was positive (not shown), a new set of primers were generated.

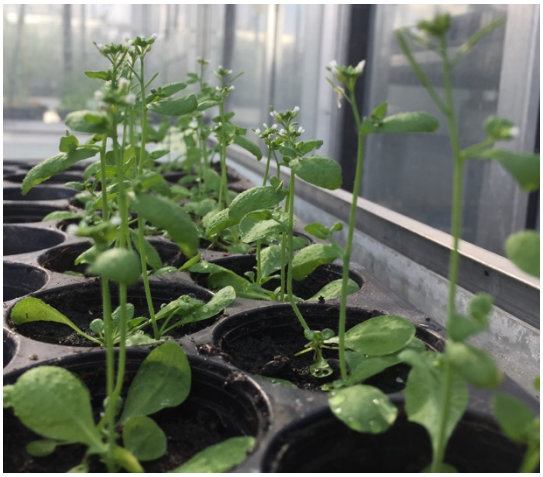


Figure 13 - *Heterozygous plants abi1-1p:RGA:GFP line 19 ready for DNA extraction*

A new DNA extraction and PCR with newly designed primers art106 and art107 was made. Results showed that the new primers were suitable for the genomic amplification of the ABI1 locus (figure 14 and 15).

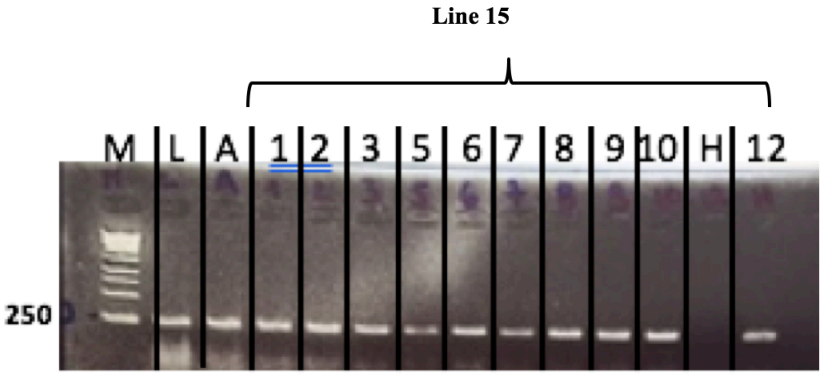


Figure 14 - *PCR before digestion of the samples from line 15 (Ler, abi1,1-12, H2O) abi1-1p:RGA:GFP , the letter M stands for DNA 1kb leader and H for water in order to see if there is unspecific bands.*

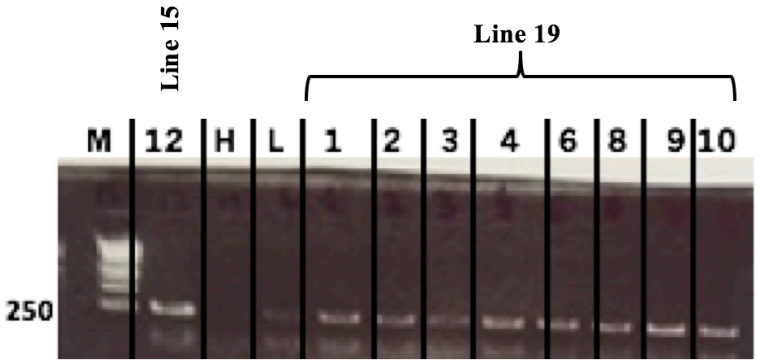


Figure 15 - *PCR before digestion from line 19 (samples ler; 1;2;3;4;6;8;9;10; H2O) and line 15 (sample 12) abi1-1pRga:GFP. the letter M stands for DNA 1kb leader and H for water in order to see if there is unspecific bands.*

Figure 16 illustrates the expected bands after the digestion with Nco I restriction enzyme.

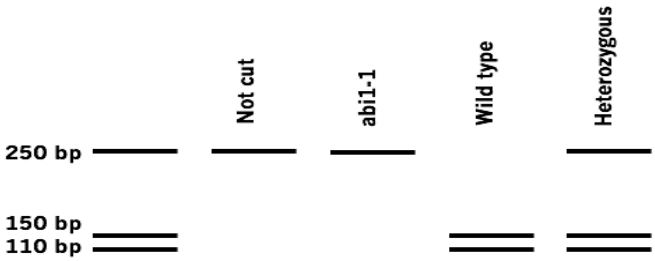


Figure 16 - *Illustration of what bands should appear on the gel after the digestion with the enzyme NcoI.*

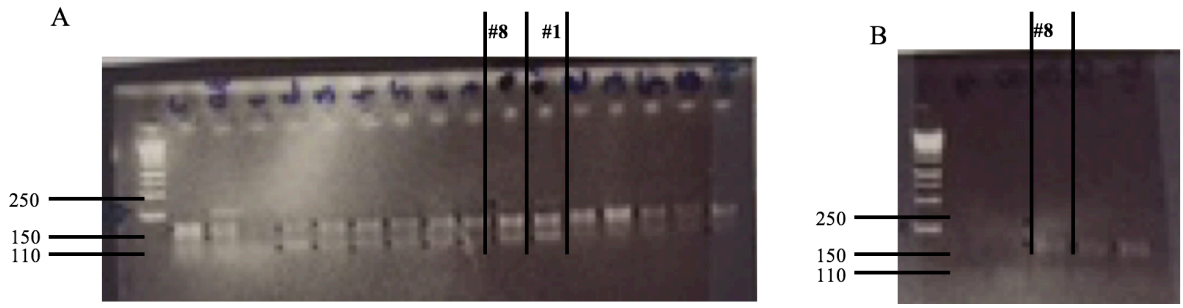


Figure 17 - *PCR after the 3h of digestion at 37ºC. There was possible to find 3 possible homozygous line 15 (sample #8) line 19 (samples #1; #8).*

The gel obtained after the digestion with NcoI (figure 17) shows that all individual positively tested were still heterozygous. Because the *abi1-1* mutation is dominant, two lines were moved forward for western blot experiments.

Seeds from line 15 #8 and line 19 #1 were harvested individually, grown on plates with MS together with genotypes wild type (non-transgenic), *pRGA:RGA:GFP* (wild type background) and *aba1-1 pRGA:RGA:GFP* (figure 18)

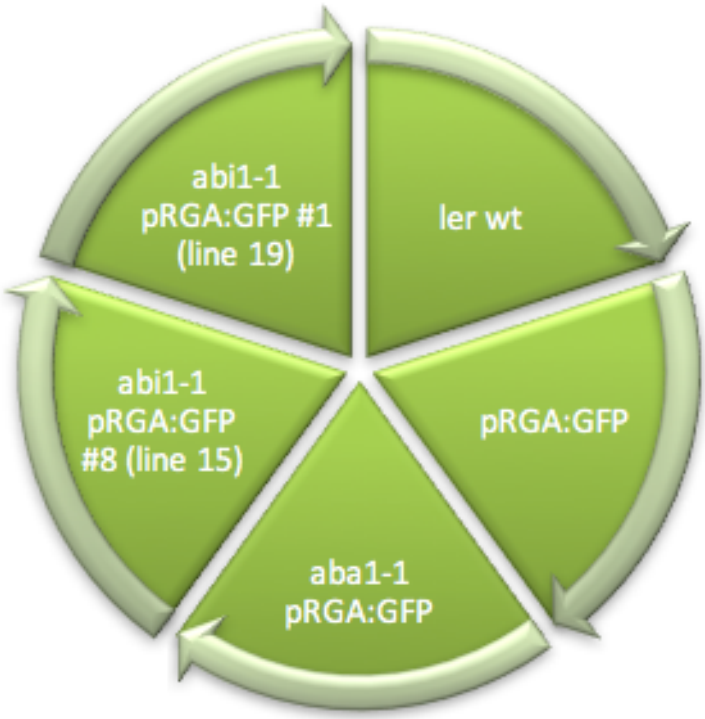


Figure 18 - *Schematic representation of a plate with MS, where the seedlings of the 5 genotypes were put to grow.*

To study the accumulation of DELLA proteins in the different ABA-related backgrounds, a western blot was performed on the five genotypes illustrated on figure 18.

Table 4 - *Weight tissue (g) and quantity of Buffer E+Pic (µl) used in each tube for protein extraction.*

Genotype	Weight (g)	Buffer E+Pic (µl)
1- LER WT	**0,07**	**70**
2- LER WT	0,05	50
3- pRGAxGFP	**0,09**	**90**
4- pRGAxGFP	0,08	80
5- aba 1-1 pRGAxGFP	**0,08**	**80**
6- aba 1-1 pRGAxGFP	0,01	10
7- abi 1-1 pRGAxGFP #1	**0,07**	**70**
8- abi 1-1 pRGAxGFP #1	0,07	70
9- abi 1-1 pRGAxGFP #8	**0,06**	**60**
10- abi 1-1 pRGAxGFP #8	0,06	60

The genotypes indicated with the numbers 1/3/5/7/9 on table 5, were chosen for western analysis, because they had similar protein concentrations.

Table 5 - *Parameters for the loading gel, calculated in an excel file with the results from the reading on spectrophotometer. The corresponding µl is the amount of protein that is loaded in the gel.*

Genotypes	A595	A595 BSA 100 µg/ml	[mg/ml]	Dilution factor	Final [mg/ml]	ug protein to load	Corresponding µl
Ler wt	0,233	0,1	2,3	0,75	1,75019692	20	11,4
pRGA:RGA-GFP	0,242	0,1	2,4	0,75	1,815551817	20	11,0
aba1-1 pRGA:RGA-GFP	0,231	0,1	2,3	0,75	1,73515475	20	11,5
abi1-1 pRGA:RGA-GFP #1	0,281	0,1	2,8	0,75	2,110060759	20	9,5
abi1-1 pRGA:RGA-GFP #8	0,265	0,1	2,6	0,75	1,987283695	20	10,1

Table 6 - *Average of the results, BSA 100 and BSA 200 controls.*

BSA100	0,109384843
BSA200	0,190634373

For western blots 20 µg of total proteins for each line was loaded onto the SDS gel (figure 19).

This showed detection of RGA:GFP protein bands in the first three wells containing *pRGA:RGA:GFP*, *aba1-1 pRGA:RGA:GFP* , and *abi1-1 pRGA:RGA:GFP* #1, respectively. I was expecting not to detect signals in the wild type control, but not in the *abi1-1 pRGA:RGA:GFP* #18.

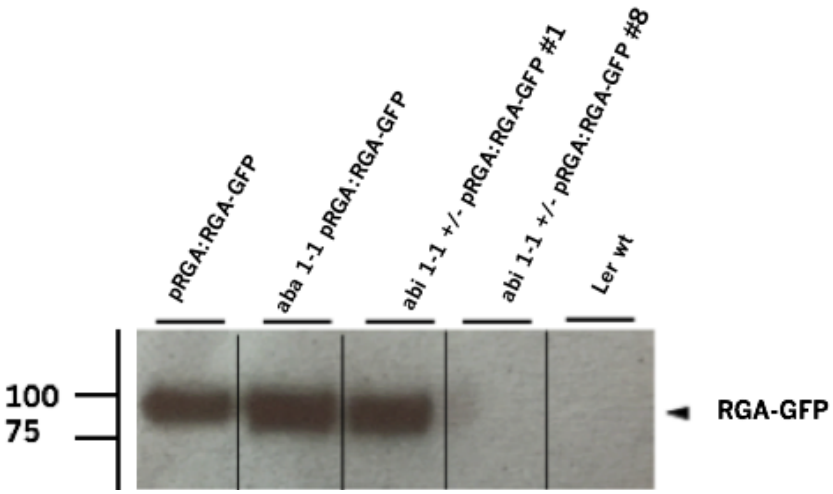


Figure 19 - *Results of the western blot between a homozygous aba mutant and an heterozygous abi mutant lines.*

As expected the wild type *Ler*, did not show any band because it lacked the transgene. In the genotype *abi1-1 pRGA:RGA:GFP* #8 I was expecting similar results to *abi1-1 pRGA:RGA:GFP* #1. The absence of detection had to do with the loading, as the well correspondent to the line *abi1-1 pRGA:RGA:GFP* #8 did not show any amount of protein as confirmed by a Ponceau assay.

In any case, from the results obtained, it was possible to observe a significant increase in the amount of RGA:GFP protein in the *abi1-1 pRGA:RGA:GFP* #1 heterozygous line, which was similar to *aba1 pRGA:RGA:GFP*. The gene ABA1 is involved in ABA biosynthesis whereas the gene ABI1 is involved in the ABA signaling pathway. Based on these results of accumulation of the protein RGA:GFP, it might be possible to conclude that ABA promotes the down regulation of RGA accumulation, one of the DELLA proteins. These effects could be interpreted either in terms of ABA promoting RGA degradation or ABA impairing the transcriptional activation of the RGA gene (something that can be tested by expression studies). To further confirm that this hypothesis is valid, the same experiments will be conducted in the homozygous *abi1-1 pRGA:RGA:GFP* plants.

3.3 Genetic interaction between GA and ABA

The regulatory role of ABA on DELLA proteins was next studied with a genetic approach. Data obtained have shown that RGA (one of the 5 DELLA proteins of *Arabidopsis*) accumulates at higher levels in ABA-deficient mutants or in mutants impaired in ABA signaling. It is thus plausible that by knockings out the *DELLA* genes we would be able to rescue growth defects of the ABA deficient background.

To begin, it was decided to cross mutant lines with the objective to obtain double and triple mutants. The crosses made were the following:

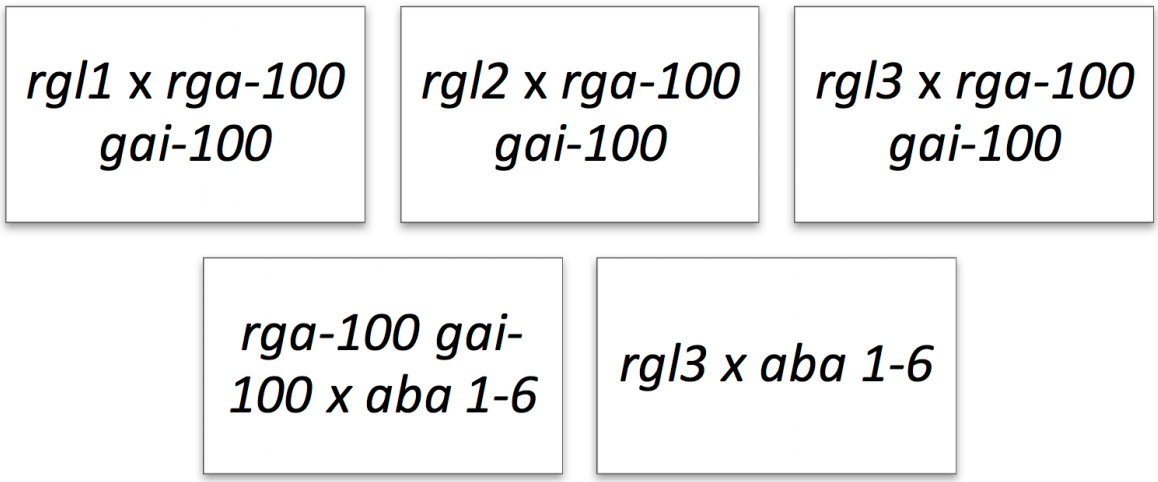


Figure 20 - *Schematic presentation of all genotypes crossed.*

The crosses were isolated individually, and seeds collected plated onto agarose medium (in case of herbicide selection) and later transferred on soil. Leaves from individual plants for each cross was collected for DNA extraction and PCR.

The first attempt of PCR was to see if all the crosses worked, with the correct primers already explained in the materials and methods section. The results confirmed all lines as being heterozygous, which means that all crosses were successful.

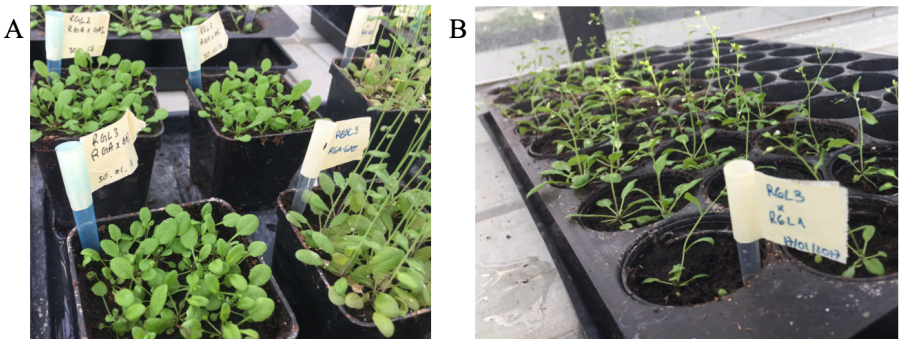


Figure 21 - *Seeds were collected and the mutants were selected by Basta resistance. The seedlings from genotypes rgl2xrga:gai and rgl3xrga:gai were transferred on soil (A) for posterior PCR analyses individually on each plant. Genotype rlg3xrgl1 was only to reproduce and use as a control (B).*

A different approach was used in genotypes with *aba1-6*. To isolate the possible homozygous *aba1* plants, we took advantage of the fact that aba mutants display yellow cotyledon (as a result of a deficiency in carotenoid production). This phenotype is visible for a limited temporal window (2-3 days), but allowed us to transfer putative homozygous plants to soil.

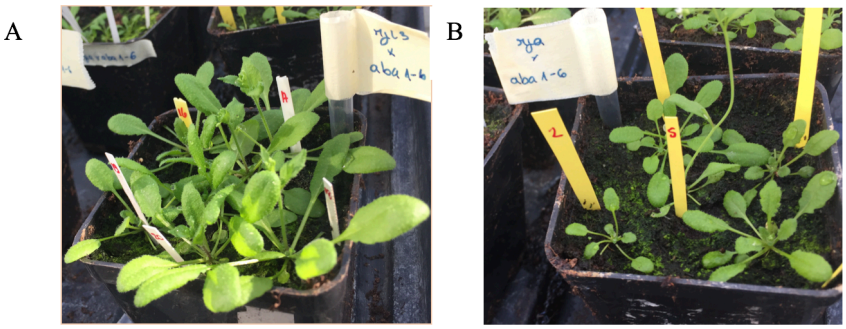


Figure 22 - *(A) Seedlings from genotypes rgl3xaba1-6 and (B) rgaxaba1-6 ready for DNA extraction in individual plants*

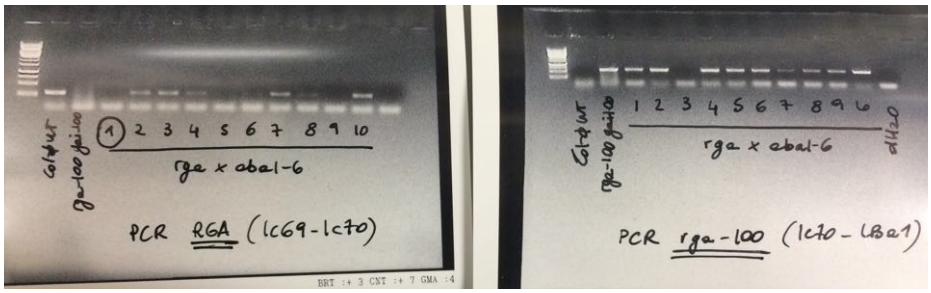


Figure 23 - *PCR analysis on samples form rgaxaba1-6 genotype, by the results we could conclude that the homozygosity was fixed on the rga-100 gai-100*

In my work, I was able to produce the F1 generation of the crosses outlined in Fig 20 and to genotype the segregating populations with the aim to identify useful double mutant combinations. This would pave the way to the generation of higher order mutants with the final goal of knocking out all the five *DELLA* genes in the *aba1* background.

4 Conclusion and Perspectives

Data obtained in my project have shown that RGA (one of the 5 DELLA proteins of Arabidopsis) accumulates at higher levels in ABA-deficient mutants or in mutants impaired in ABA signaling. The conclusion of these experiment, is that ABA impairs the accumulation of DELLA proteins, through an unknown mechanism. Follow up experiments will ascertain whether the stability of RGA protein is increased in ABA-defective mutants, because of reduced GA production or signaling. The pattern of RGA accumulation will be studied in the presence of Cycloheximide, a potent protein synthesis inhibitor. In this way, the half-life of RGA protein accumulation can be precisely evaluated in the ABA-deficient background compared with the wild type.

The next step is to test also the hypothesis that higher levels of RGA protein might derive from more *RGA* transcript accumulating being accumulated the different ABA-deficient backgrounds compared with the wild type. This will be done by expression analysis, using RT-qPCR approaches. I will then extend the analysis to other DELLA genes to understand whether all the *DELLA* genes are transcriptionally regulated in response to varying levels of ABA.

ABA might affect the rates of GA biosynthesis or degradation. Hints about this potential mechanism will be gained by measuring the transcript levels of key GA biosynthetic genes, known to have a prominent role in the accumulation of GA in plants. However, conclusive evidence in support of alterations in GA levels might ultimately derive from direct measurements of GA in different ABA-defective backgrounds.

Other hypothesis that will be interesting to test is whether and to what extent ABA acts on GA to promote growth. There are some phenotypic similarities between ABA and GA deficient mutants as they both appear dark green, with compact in size and late flowering. Based on previous observations of increased DELLA accumulation in ABA-deficient mutants, one might wonder whether the ABA-related phenotype depends on alterations in GA signaling. We plan to test this hypothesis genetically by systematically knocking out all the DELLA genes in an ABA-deficient background. If DELLA over-accumulation contributes to the reduced growth phenotype of ABA-deficient mutants, then it means that ABA can also be involved in the activity of DELLA proteins.

Technically speaking this experiment is straightforward but involves the generation of a sextuple mutant, the construction of which will require more than one year. As a parallel experiment, we will spray GA onto ABA-deficient mutant with aim to recover the growth defects which might depend on impaired GA accumulation. This of course, assumes that ABA does not affect GA signaling, and the rate of DELLA degradation (an objective detailed

above). The genetic data and the exogenous GA spray experiments will be generally used to understand what aspects of ABA depend on GA signaling and which are independent.

Once all the above questions will be answered and supported by data this project will lead to a substantial improvement of the scientific knowledge. We believe that the results from this project will provide breakthroughs in Arabidopsis that could be applied to other studies, and change what is known about the degradation of DELLA protein in plants by revealing a new facet of the ABA-GA cross talk.

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SUPPLEMENTARY DATA

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Annex A – Sequences

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aba1-6

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actaaaaggtaattatattatcaaattgtttactttttctt

the aba 1-6 allele show a change in aa nº160 Gly -> Ser, and after this position the strand suffered a modification in 478 nucleotides. (G -> A TRANSITION)

PCR ABA1-6 (mt115/mt116)

Primers

Forward =GCTCGGAGTAAAGGCGGCGA
Reverse =GGCGTCACGGGGACTTCCTG
94ºC 1'
94ªC 15'' = Denaturation (35 cycles)
55ºC 30'' = Elongation (35 cycles)
72ºC 35'' = Annealing (35 cycles)
72ºC 5''
15ºC (..)

Digestion with the restriction enzyme BSAI (10microliters)
Sense strand ABA1 320bp /160bp
Anti-sense strand aba 1-6 480bp

BSA 1mg/ml= 0,3 microliters
BSAI = 0,4 microliters

AT4G26080 – ABI1

cataaatagtagattcgaagcaattgttgcattagcctacccatttcctccttctttctctcttctatctgtgaacaaggcacattagaactcttctttt
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abi1-1_GENOTYPING

ABI1

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abi1-1

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NcoI CCATGg

Lcm48 caccATGGAGGAAGTATCTCCGGCG
Mr130 AGTTCAAGGGTTTGCTCTTGAG

ABI1_Protein sequence

MEEVSPAIAG PFRPFSETQM DFTGIRLGKG YCNNQYSNQD SENGDLMVSL PETSSCSVSG
SHGSESRKVL ISRINSPNLN MKESAAADIV VVDISAGDEI NGSDITSEKK MISRTESRSL
FEFKSVPLYG FTSICGRRPE MEDAVSTIPR FLQSSSGSML DGRFDPQSAA HFFGVYDGHG
GSQVANYCRE RMHLALAEEI AKEKPMLCDG DTWLEKWKKA LFNSFLRVDS EIESVAPETV
GSTSVVAVVF PSHIFVANCG DSRAVLCRGK TALPLSVDHK PDREDEAARI EAAGGKVIQW
NGARVFGVLA MSRSIGDRYL KPSIIPDPEV TAVKRVKEDD CLILASDGVW DVMTDEEACE
MARKRILLWH KKNAVAGDAS LLADERRKEG KDPAAMSAAE YLSKLAIQRG SKDNISVVVV
DLKPRRKLKS KPLN*

ABI1→abi1-1 = G180→D180 = GGC→GAC

ABI1 ORF

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abi1-1 ORF

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GAI_AT1G14920

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Primers:
Forward = CACCATGAAGAGAGATCATCATC
Reverse = ATTGGTGGAGAGTTTCCAAG
gai-100 (SAIL_587_C02)

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CTCTCCACCAATTAGatggtggctcaatgaattgatctgttgaaccggttatgatgatagatttccgaccgaagccaaactaaatcctactgtttttccc
Lc81
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atgactaatgagcctgattaaactgtctcttatgataatgtgctgattctcatgaatatgctcttttaatgtgcatggtattataggtggaccagattat

PCR gai-100
(lc80/lc81)
94ºC 1'15''
94ªC 15'' = Denaturation (35 cycles)
55ºC 25'' = Elongation (35 cycles)
72ºC 1'40'' = Annealing (35 cycles)
72ºC 5'
15ºC (..)

Primers : lc80 + lc81 = 1604 bp
Foward (lc80) = caaccATGAAGAGAGATCATCATC / Foward GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC

Reverse (lc81) = CTTGGAAACTCTCCACCAATTAG / ATTGGTGGAGAGTTTCCAAG

RGL1_AT1G66350.1

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CCCGCCAAGCC**<-SALK**
AAGACCGAGTGATGTCGGAGTTATTCCTAGGACGGCAGATACTAAACCTTGTGGCATGCGAAGGAGAAGACCGGG
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ttggttttcgataaa

Salk? NASC ID: N16353
RB TGATAGTGACCTTAGGCGACT TTTGAACGC
Lcm 110 Fw = CATCAATGACGACGGT
Lcm 111 Re = TTATTCCACACGATTGATTCGCCA (TGGCGAATCAATCGTGTGGAATAA)

RGL2_AT3G03450

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Rgl2-1

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lcm42 Fw = GAGCTCCACCGGTTATTAGCCC

lcm41 Re = GGCGAGTTTCCACGCCGAGG (CCTCGGCGTGGAAACTCGCC)

LB1 (5'-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3')
LB2 (5'-GCTTCCTATTATATCTTCCCAAATTACCAATACA-3')
LB3 (5'-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3')

RGL3_ AT5G17490.1

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SAIL_349_B10

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lcm 112 Fw = CTGGCTCAAGCCATCGGCGTC

lcm 113 Re = CAATGAAAACCTCTCAAAAAACCC

complementary Re = (GGGTTTTTTGAGAGGTTTTCATTG)

LB TGGTTCACGTAGTGGGCCATCG

rgl3-1(N873377) SAIL_349_B10