AUTÓNOMA DE MADRID UNIVERSITY

Molecular Biology Department

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Target destabilization and chromatin remodelling are coordinated by CRL4-CDDD E3 ubiquitin ligases to repress photomorphogenesis in *Arabidopsis thaliana* (L.)

PhD thesis
submitted by

Amr Abdelmotagaly Nassrallah

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Summary.

Plants adapt their growth and development according to the environmental light information. Light signals are perceived, processed and integrated through highly structured intracellular networks. These rely on tight transcription coordination, achieved by chromatin remodeling synchronized with transcription factor binding to DNA, and precise control of their activity and abundance. Control of transcription factor levels is mediated, in many cases, by specific ubiquitination and degradation at the 26S proteasome. Protein target ubiquitination is mediated, among others, by Cullin 4 RING E3 ubiquitin Ligases (CRL4s) associated to COP10-DDB1-DET1-DDA1 (CDDD) complexes. Recently, we have characterized DDA1 as a novel type of substrate adaptor for CRL4-CDDD (Irigoyen *et al*., 2014). In a yeast two hybrid screening, we found SGF11 as a DDA1 interactor. SGF11 is a member of the deubiquitination module (DUBm), which in yeast and animals has been shown to be part of the SAGA (SPT-ADA-GCN5-Acetyltransferase) complex. The role of DUBm on H2Bub (ubiquitinated histone 2B) deubiquitination to promote transcription elongation is well described in yeast, *Drosophila* and mammals. However, in plants, the function of DUBm remains unexplored.

In this study, we show that the *Arabidopsis* DUBm is composed of at least three subunits: SGF11, ENY2 and UBP22, with UBP22 as the H2B ubiquitin-specific protease. According to their predicted function, loss of SGF11 or UBP22 function increased the abundance of H2Bub. DUBm function is likely controlled by the light conditions since all three components were destabilized under dark conditions. We demonstrate that SGF11 degradation involves DET1 function, suggesting that upon recognition of SGF11 by DDA1, CRL4-CDDD E3 ligases promote its ubiquitination and proteasomal degradation. In agreement with this notion, CRL4-CDDD function alters global H2Bub abundance.

Phenotypic characterization of *sgf11* and *ubp22* mutants showed that the DUBm also plays a role in the control of plant responses to light. Thus, both *sgf11* and *ubp22* mutations synergistically enhanced the detiolated phenotype of *det1-1* plants under dark conditions, whereas SGF11 overexpression ameliorated *det1-1* photomorphogenic defects. Photomorphogenic defects correlated, respectively, with increased or reduced abundance of HY5, a positive regulator of light responses whose accumulation is repressed by CRL4-CDDD E3 ligases. Altogether, our findings indicate that the DUBm acts as a negative regulator of photomorphogenesis, and point to a role for H2B deubiquitination in this process. We propose a model in which de DUBm facilitates recruitment of CRL4-CDDD complexes to light responsive

promoter regions in order to increase recognition and targeted degradation of HY5 to repress photomorphogenic development.

Abbreviations.

3AT: 3-amino-1,2,4-triazol.

A. thaliana: Arabidopsis thaliana.

A. tumefaciens : Agrobacterium tumefaciens.

bHLH: basic helix loop helix.

BiFC: Bimolecular fluorescence complementation.

CAB2: Chlorophyll A/B binding protein 2.

CCA1: Circadian Clock Associated 1.

CDDD: COP10-DDB1-DET1-DDA1.

cDNA: Complementary DNA strand.

CLX: Cycloheximide.

CO: CONSTANS.

CoIP: coinmunoprecipitation.

Col: *Arabidopsis thaliana* ecotype Columbia-0.

COP1: CONSTITUTIVELY PHOTOMORPHOGENIC 1.

CRL4s: Cullin 4 RING Ligases.

CRL4s: Cullin 4 RING Ligases.

CRY1, -2: Cryptochrome 1 and 2.

CSN: COP9 signalosome.

CUL4: cullin 4.

DAPI: 4,6-diamidino-2-fenilindol.

DDA1: DDB1, DET1-Associated 1.

DET1: DE-ETIOLATED1

DTT: Dithiothreitol.

DUBm: deubiquitination module.

E. coli: Escherichia coli.

ABBREVIATIONS

ELF3: EARLY FLOWERING 3.

ELF4: EARLY FLOWERING 4.

ENY2: Enhancer Of Yellow 2.

FAR1: FAR RED IMPAIRED RESPONSE 1.

FHY3: FAR RED ELONGATED HYPOCOTYL 3.

FKF: FLAVIN BINDING KELCH REPEAT.

FR: Far red.

FT: FLOWERING LOCUS T.

GA: gibberellin.

GCN5: GENERAL CONTROL NON-REPRESSIBLE 5.

GFP: Green fluorescent protein.

HFR1: LONG HYPOCOTYL IN FAR RED.

HY5: ELONGATED HYPOCOTYL.

IB: immunobloting.

Kan+: Kanamycin resistance.

LD: Long day.

LHY: LATE ELONGATED HYPOCOTYL.

LKP2: LOV KELCH PROTEIN 2.

MS: Murashige & Skoog media.

MSMO: MS-minimal organics media.

N. benthamiana: Nicotiana benthamiana.

PBS-T: PBS con 0.1% tween-20.

P_{fr}: far-red light absorbing form of the phytochrome.

PHOT1-2: PHOTOTROPINS.

PHYA-E: PHYTOCHROMES.

PIF 1-7: PHYTOCHROME INTERACTING FACTORS.

PIL1: PHYTOCHROME INTERACTING FACTOR3-LIKE1.

P_r: red light absorbing form of the phytochrome.

qPCR: quantitative polymerase chain reaction.

RPT5: 26S PROTEASOME AAA-ATPASE SUBUNIT.

S. cerevisiae: Saccharomyces cerevisiae.

SAGA: SPT-ADA-GCN5-Acetyltransferase.

SAS: Shade avoidance syndrome.

SCF: complejo proteico SKP1-CDC53/CUL1-F-BOX.

SCF^{SLY1}: E3 Ubiquitin ligase complex (Skip1, Cullin, F-box).

SD: Short day.

SGF11: SAGA associated factor 11.

Spe+: Spectinomycin resistance.

TAP: tamdem affinity purification.

TOC1: TIMING OF CAB EXPRESSION 1, also called PRR1.

Ub: ubiquitin.

UBP22: ubiquitin-specific protease 22.

UVR8: UVB-RESISTANCE .8

ZT: *Zeitgeber* time or lights-on signal.

ZTL: ZEITLUPE.

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INTRODUCTION

Introduction.

Plants rely on complex regulatory networks that allow them to integrate different but simultaneous environmental and stress signals and to translate them into coordinated adaptive responses. Knowledge on this regulatory networks is of key importance because it can help to design crops with improved tolerance to several stress conditions, one of the actual goals towards achieving food security (Mittler and Blumwald. 2010). To adapt to such adverse situations, plants have evolved sophisticated mechanisms that help them to perceive developmental and stress signals and to trigger adaptive responses that enable plant growth, survival and reproduction. Complexity of these responses aids surviving harsh external conditions also anticipating changes to a wide array of abiotic and biotic stresses. During the last decade, the importance of protein ubiquitination in the control of developmental and stress responses in plants has been demonstrated (Vierstra, 2009). Ubiquitin (Ub) conjugation to proteins (i.e. ubiquitination) may trigger proteasomal degradation of protein targets or changes in their properties (e.g., protein activity, localization, assembly and interaction ability), depending on specific Ub chain configurations (Hershko and Ciechanover, 1998; Ikeda and Dikic, 2008; Deshaies and Joazeiro, 2009).

Ubiquitination is mediated by an enzymatic cascade in which E3 Ub ligases (E3) provide the substrate specificity. Cullin RING Ligases (CRLs), the largest class of E3s in plants, represent a family of modular complexes, consisting of at least seven different cullin scaffold proteins, each of them serving as a building block for the assembly of tens or more multi-subunit CRLs (Deshaies and Joazeiro, 2009). Among this class, Cullin4 RING Ligases (CRL4) control key aspects of cell biology in eukaryotes, including cell cycle progression and DNA damage repair and replication (Jackson and Xiong, 2009; Biedermann and Hellmann, 2011). In plants, CRL4 are involved in biological processes spanning the plant´s whole life, including seedling photomorphogenesis, circadian clock function and flowering (Yu *et al*., 2008; Biedermann and Hellmann, 2011). These processes are tightly regulated by the light conditions. Indeed, CRL4 E3 ligases play a fundamental role as negative regulators of light signaling.

Chromatin remodeling complexes have been also associated to the control of plant responses to light. This is the case of the SPT-ADA-GCN5-Acetyltransferase (SAGA) complex. In plants, the SAGA complex activity has been characterized, mainly by using mutants on its histone

acetyltransferase subunit GCN5, as a remodeling complex that acts on light-responsive chromatin regions overlapping with HY5 binding sites (Barneche *et al*., 2014). However, the H2B deubiquitination module (DUBm) of SAGA has not been characterized so far in plants and whether it is also involved in the control of chromatin dynamics at light-responsive gene regions is totally unknown. In these regards, we are interested in exploring the regulatory connection between CRL4-CDDD and the DUBm.

As part of this study, we found that Arabidopsis (*Arabidopsis thaliana*) DDA1, a substrate receptor of CRL4-CDDD E3 ligases, interacts in vivo with SAGA-associated factor 11 (SGF11), a component of DUBm in yeast and mammals required for its histone deubiquitinase activity and therefore chromatin remodelling (Lang *et al*., 2011; Rodríguez-Navarro 2009). We are interested in exploring the potential regulatory connection between DDA1 and SGF11.

In this study, we provide further knowledge on CRL4-CDDD function(s) in the control of chromatin-associated processes and how they are integrated with target destabilization to control photomorphogenesis in Arabidopsis.

Light perception and signaling in plants.

Higher plants not only transform solar energy into chemical energy through the process of photosynthesis but also use light as an informational cue to control a multitude of physiological responses throughout their life cycle. Thus, light affects all aspects of plant development, including seed germination, seedling and leaf development, shade avoidance responses and flowering (Jiao *et al*., 2007). Collectively these responses are known as photomorphogenesis (Kendrick and Kronenberg, 1994). Plants have the ability to sense the quality, intensity, wavelength, direction and duration of the ambient light in order to optimize their growth and development. To sense this wide range of environmental light cues, higher plants possess multiple light sensors or photoreceptors (Somers and Fujiwara, 2009). Thus, five major classes of photoreceptors have been described in Arabidopsis: the phytochromes (phyA to E) acting predominantly in red/far-red wavelengths (Wang and Deng, 2004), the cryptochromes (CRY1 and 2) responding in blue and UVA (Yu *et al*., 2010; Chaves *et al*., 2011), the phototropins (PHOT1 and 2) and the ZTL/FKF1/LKP2 proteins, both responding to blue light, and the more recently identified UVB photoreceptors (UVR8) (Rizzini *et al*., 2011). Thus, light at specific wavelength ranges will trigger activation of different families of receptors,

leading to important changes in plant hormone homeostasis and in gene expression that will shape plant growth and development.. Indeed, the control of photomorphogenesis widely relies on the transcriptional regulation of light-responsive genes, as shown by genome-wide transcriptomic data estimating that expression of approximately one-third of the Arabidopsis genome changes in response to light (Tepperman *et al*., 2003). Accordingly, several classes of photoresponsive transcriptional activators have been identified; including bZIP proteins ELONGATED HYPOCOTYL5 (HY5) and HY5 HOMOLOG (HYH), MYB domain-containing LONG AFTER FAR-RED LIGHT1 (LAF1) and the atypical bHLH protein LONG HYPOCOTYL IN FAR-RED1 (HFR1), among others (Holm *et al*., 2002; Jang *et al*., 2005; Osterlund *et al*., 2000a; Saijo *et al*., 2003; Seo et al. 2003; Seo *et al*., 2004; Yang *et al*., 2005). Oppositely, members of the PHYTOCHROME INTERACTING FACTOR (PIF) family have been described as negative regulators of photomorphogenesis. In addition, light perception and signaling is tightly regulated by components of the ubiquitin-proteasome system (the so-called COP/DET/FUS) proteins, which modulate the stability and activity of both photoreceptors and transcriptional regulators. Coordinated function of photoreceptors, positive and negative transcriptional regulators, and the COP/DET/FUS proteins provides plant plasticity to respond to changing light conditions, which is of particular relevance during seedling development.

Photomorphogenesis versus skotomorphogenesis: The identification of the COP/DET/FUS proteins.

Upon exposure to light, seedlings follow a de-etiolated or photomorphogenic developmental pattern characterized by a short hypocotyl (primary stem at the seedling stage), expanded cotyledons (first leaves) and increased chloroplast production, in contrast to the skotomorphogenic or etiolated phenotype of plants grown in darkness (Sullivan and Deng 2003). This distinct morphological pattern was used by different research groups to identify mutants with modifications in pathways controlling the light-triggered development of plants. Several mutant screens performed using Arabidopsis as a plant model species led to the identification of a group of loss-of-function *cop* (constitutive photomorphogenic) and *det* (de-etiolated) mutants (reviewed in Wei and Deng 1996). These mutants share a constitutive photomorphogenic phenotype even when grown in darkness. It has been found that some of these mutants are allelic to a group of mutants characterized by accumulation of high levels of anthocyanins (purple pigments) and are therefore designated *fusca* (from the Latin word

meaning ‘‘purple’’). Positional cloning of the *COP/DET/FUS* genes showed that they encode components of three distinct protein complexes: the COP1-SUPRESSOR OF PHYA-105 (SPA) complex, the COP9 signalosome (CSN) and the COP10-DET1-DDB1 (CDD) complex (Lau and Deng 2012). As it is described below, these three biochemical entities belong to the plant UPS, and function as repressors of photomorphogenesis in close connection with CRL4 E3 ubiquitin ligases.

CRL4 E3 ligases function as repressors of light signaling.

CRLs represent the largest class of E3s in plants. CRLs represent a family of modular complexes, consisting of at least seven different cullin scaffold proteins, each of them serving as a building block for the assembly of tens or more multi-subunit CRLs (Deshaies and Joazeiro, 2009). Among this class, CRL4 control key aspects of cell biology in eukaryotes, including cell cycle progression and DNA damage repair and replication (Jackson and Xiong, 2009; Biedermann and Hellmann, 2011). In plants, CRL4 functional significance can be realized by the number and relevance of the processes they regulate, which span the plant´s whole life, including embryogenesis, seedling photomorphogenesis, circadian clock function and flowering (Yu *et al*., 2008; Biedermann and Hellmann, 2011). As well, CRL4s control different abiotic stress responses such as drought tolerance, nutrient deprivation and DNA damage responses (Guo *et al*., 2013). Thus, several CRL4 protein targets have been identified in plants, including positive regulators of light signaling, flowering, metabolic homeostasis, DNA damage repair and responses to stress hormone abscisic acid (ABA) (reviewed in Biedermann and Hellmann, 2011; Guo *et al*., 2013).

CRL4 use CULLIN4 (CUL4) as a scaffold protein for the rest of the complex, Ring finger protein RBX1 for Ub conjugase (E2) recruitment and Damaged-specific DNA Binding protein1 (DDB1) for interaction with substrate receptors, namely DCAFs (DDB1 and CUL4-associated factors) that usually contain WDxR motifs and recognize specific targets for ubiquitination. In Arabidopsis there are two genes encoding DDB1 proteins, DDB1A and DDB1B. Although their products are 91% identical they have partially distinct functions and may interact with different DCAF subsets to mediate ubiquitination of specific protein substrates in each case (Schroeder *et al*., 2002). There are about 80 WDxR-containing DCAF proteins in Arabidopsis and rice, although the function of just a few of them has been characterized in depth

(Biedermann and Hellmann, 2011). Well-characterized DCAF proteins include CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) and SUPPRESSOR OF PHYA (SPA) proteins. As it is described in the following section, the latter act under dark conditions as substrate adaptors of CRL4 E3 ligases to recognize and promote degradation of different photoreceptors and transcription factors.

COP1 as a negative regulator of light signalling.

In plants, the function of COP1 is tightly close to light signaling pathways. COP1 acts as a central negative regulator of photomorphogenesis by inducing skotomorphogenesis in the dark, where it promotes the ubiquitination and degradation of various photomorphogenesis-promoting factors. COP1 functions as part of a protein complex, as in the dark it associates with SPA proteins and together constitute a substrate adaptor module for CRL4 E3 ligases (Figure 1d; $CRL4^{COP1\text{-}SPA}$). The family of SPA proteins consists of 4 members in Arabidopsis (SPA1-4), although only two SPA subunits, together with a COP1 homodimer, are found in $CRL4^{COP1\text{-}SPA}$ complexes. These E3 ligases target transcription factors, such as HY5, HYH, HFR1, and LAF1, and phyA and CRY2 photoreceptors for degradation via the 26S proteasome, leading to suppression of photomorphogenesis under dark conditions (Figure 1; Casal *et al*. 2014). Besides control of seedling development, COP1 plays an integral role in other plant processes, most of which are light-regulated, including flowering (Liu *et al*., 2008), circadian clock function (Yu *et al*., 2008), shade avoidance responses (Crocco *et al*., 2010) and UV-B signaling (Favory *et al*., 2009).

COP1 contains a RING finger motif, a coiled-coil domain, and a WD-40-repeat domain, that facilitate interaction with other proteins. However, COP1 interacts with most of its substrates via its WD-40 domain (Yi and Deng 2005). COP1 and the four SPA proteins (SPA1-SPA4) share further structural similarity in that they contain related C-terminal WD-repeat domains which have dual roles in substrate recruitment and binding of DAMAGED DNA-BINDING PROTEIN1 (DDB1) of the CUL4 complex. However, in their N-termini, SPA proteins contain a kinase-like domain instead of a RING finger motif (Hoecker *et al*., 1999). Within $CRL4^{COP1\text{-}SPA}$ complexes, COP1 holds ubiquitination activity towards specific protein targets, whereas SPA proteins do not display such activity. However, it has been shown that SPA function is required for ubiquitination of substrates by $CRL4^{COP1\text{-}SPA}$, since mutation of *Arabidopsis SPA1* impairs COP1

E3 ligase activity, likely by affecting assembly of the $CRL4^{COP1\text{-}SPA}$ complex (Seo *et al.*, 2003).

On the contrary, COP1 can act as a positive regulator for signaling of specific light wavelengths. Thus, when plants are exposed to UV-B light, the UV-B photoreceptor UVR8 (UV RESISTANCE LOCUS8) suffers a conformational change that enables interaction with the COP1-SPA module, which is released from the $CRL4^{COP1\text{-}SPA}$ complex. Such association converts COP1-SPA from a repressor to a promoter of photomorphogenesis. Thus, sequestering of COP1-SPA by UV-B-activated UVR8 increases HY5 stability and activity, which leads to UV-B-induced gene expression and a photomorphogenic developmental pattern (Figure 3). (Lau and Deng 2012, Kong and Okajima 2016).

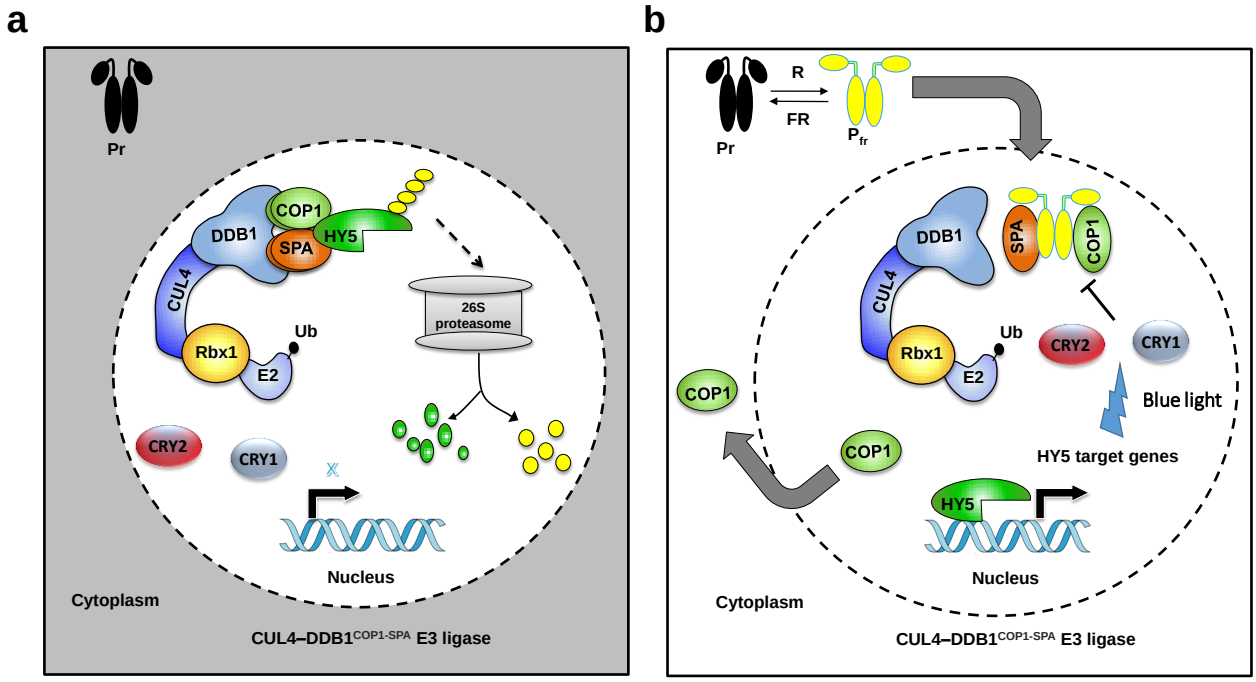


Figure 1: COP1 regulation of HY5 levels in (a) dark and (b) light. In the dark, nuclear localized COP1-SPA targets HY5 for degradation via the 26S proteasome and prevents photomorphogenesis. In the light, the active Pfr form of phytochrome enters the nucleus and inhibits COP1-SPA interaction. In addition, COP1 is slowly exported from the nucleus. This results in HY5 accumulation, expression of HY5 target genes and light growth. (adapted from Fernando and Schroeder. (2015))

COP1 regulation of photoperiodic signaling and flowering transition.

In *Arabidopsis*, the transition from vegetative to the flowering stage is largely controlled by day length (photoperiod). Thus, *Arabidopsis* is a facultative long day plant which flowers earlier in

long days (LD) than it does in short days (SD). CONSTANT (CO) protein acts as a central regulator of photoperiodic flowering and its abundance directly correlates with the timing of flowering. CO is precisely regulated at both transcriptional and post-translational levels, and this is crucial for *Arabidopsis* to discriminate the photoperiod and response to light. Thus, *CO* gene expression is regulated by the circadian clock and CO protein stability and activity are regulated by light. Indeed, during the day, CO protein is stabilized, whereas at night CO protein is rapidly degraded through the 26S proteasome pathway. In this way, under LD conditions, peak expression of *CO* gene in phloem companion cells coincides with external light signaling, which allows CO protein stabilization and activation of its target gene *FLOWERING LOCUS T* (*FT*). Then, newly synthetized FT protein moves through the phloem to induce flowering at the shoot apex (Jang *et al*., 2008). However, in SD-grown *Arabidopsis* plants, peak expression of *CO* gene overlaps with the night period, which triggers CO protein destabilization in a COP1-dependent manner. Indeed, under dark conditions, COP1 is able to directly interact with the C-terminal region of CO, COP1-CO interaction promotes ubiquitination and degradation of the latter. (Jang *et al*., 2008). For this, mutations in COP1 cause an early-flowering phenotype under SD, which is largely related to the change of CO abundance. (Imaizumi and Kay 2007). SPA proteins are likely involved in this process, since *spa1 spa3 spa4* triple mutants exhibit strongly increased CO protein levels, and display an early-flowering phenotype, indicating that SPA proteins negatively modulate CO abundance. The mechanism by which CO protein is stabilized by light involves the blue light photoreceptors CRYs. Thus, in response to blue light, CRYs photoreceptors suppress COP1 activity by dissociating the formation of COP1-SPA complex, thereby repressing its E3 ubiquitin ligase activity towards CO (Lian *et al*., 2011 and Zuo *et al*., 2011).

COP1 modulation of circadian clock function.

The daily oscillation of *CO* gene expression is regulated by circadian clock-associated components, including GIGANTEA (GI), FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1), CYCLING DOF FACTORS (CDFs) and EARLY FLOWERING 3 (ELF3) (Fornara *et al*., 2009). FKF1 has a dual role in this process acting as a blue light photoreceptor through its LOV domain and being part of CRL1 E3 ubiquitin ligases, as it also contains an F-box domain. Under light conditions, FKF1 forms a complex with GI in a light-dependent manner, which contributes to control the *CO* transcript level by mediating the degradation of *CO* transcriptional repressors,

CDFs (Fornara *et al*., 2009). When lights are off, FKF1-GI is inhibited by GI destabilization mediated by COP1. In this process, ELF3 acts as a substrate adaptor to allow COP1-GI interaction, which facilitates GI ubiquitination by COP1 (Yu *et al*., 2008) Subsequent degradation of GI at the proteasome results in the disassociation of the FKF1-GI complex, accumulation of CDF repressors and negative regulation of *CO* expression (Rubio *et al*., 2009).

$CRL4^{COP1\text{-}SPA}$ and PIFs collaborate to repress photomorphogenesis.

The bHLH type transcription factors PIFs function as negative regulators of photomorphogenesis, acting downstream of phytochromes to promote skotomorphogenesis. In *Arabidopsis*, PIFs protein family contains seven members (PIF1/PIF3-LIKE 5, PIF3, PIF4, PIF5/PIL6, PIF6/PIL2, PIF7, and PIF8) that accumulate in the dark and act redundantly to promote dark growth. Thus, whereas single mutations in PIF members cause little effect in seedling development, a quadruple *pifq* (which lacks PIF1, PIF3, PIF4 and PIF5) displays a strong *cop*-like phenotype (Leivar and Monte 2014). However, some functional specificity has been reported within the PIF family, as PIF1 seems to play a major role in repressing seed germination. Interestingly, direct interaction between COP/DET/FUS components and PIFs has been shown. Indeed, it was found that PIF1 interacts with COP1, SPA1 and HY5, and facilitates HY5 degradation by maximizing the affinity of COP1 to HY5. Thus, PIFs play a role in substrate recruitment and ubiquitination by $CRL4^{COP1\text{-}SPA}$ participating therefore in negative regulation of photomorphogenesis under dark conditions (Xu *et al*. 2014).

Upon transition to light conditions, phytochromes (Phys) are photoconverted into their Pfr active forms which accumulate in the nucleus and directly interact with PIFs. Active Phys phosphorylate PIF proteins, which makes them susceptible of ubiquitination and degradation via the proteasome (Figure 2a and 2b). PIF ubiquitination might be mediated also by COP1, as it was shown that PIF1 is destabilized in a COP1-dependent manner, although the action of other unknown E3 ligase(s) cannot be discarded.

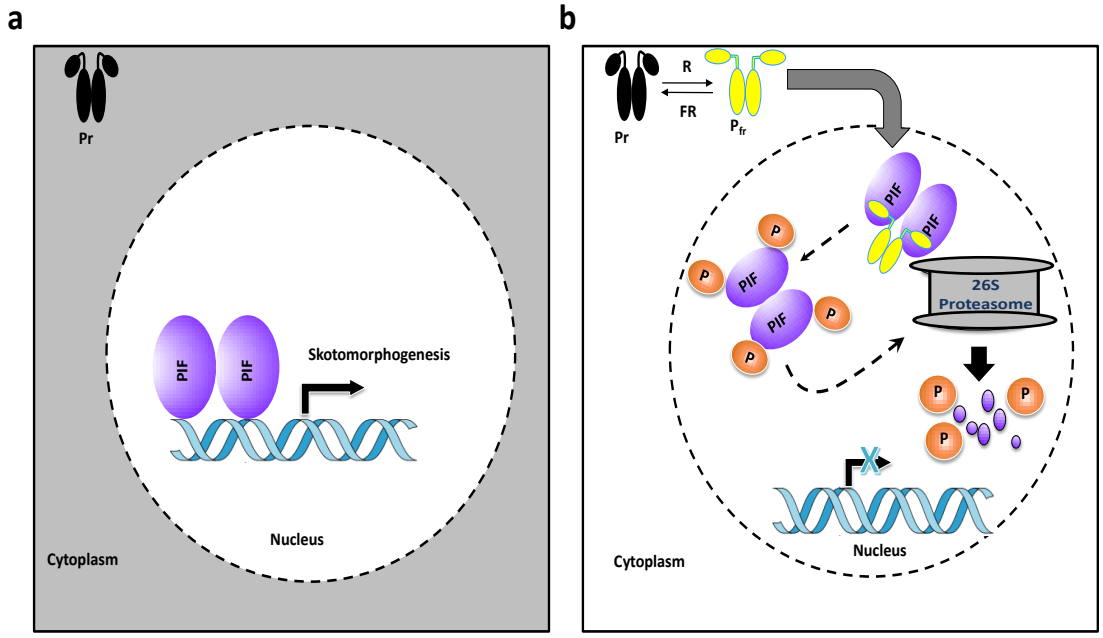


Figure 2: Interaction between light and PIFs in light signaling. (a) In the dark phytochromes are in the biologically inactive Pr form and are localized in the cytosol. Homo and heterodimers of PIFs bind to light regulated genes, preventing their expression and repressing photomorphogenesis. (b) In the light, the active Pfr form of phytochromes move to the nucleus to bind and rapidly phosphorylates PIFs. The phosphorylated PIFs are degraded via the 26S proteasome. (adapted from Fernando and Schroeder. (2015)).

Inhibition of COP1 by the photorreceptors.

Under dark conditions, COP1 accumulates in the nucleus where it associates with SPA proteins as part of $CRL4^{COP1\text{-}SPA}$ complexes that target positive regulators of photomorphogenesis for degradation, including HY5, HFR1, LAF1, FHY3, phyA and CRY2. On the contrary, under light conditions, phytochrome (phy) photoreceptors enhance light development by disrupting the interaction between COP1 and SPA, thereby allowing photomorphogenesis to proceed. The mode of action of phys to inactivate COP1-SPA has remained unclear until recent date (Li *et al*., 2011). Thus, it was recently shown that phyA and phyB directly interact with SPA proteins and that this interaction likely triggers COP1-SPA inactivation (Sheerin *et al*. 2015). In the presence of light, both phyA and phyB inhibited COP1/SPA1 interaction and disassociated them from the rest of the CRL4 E3 ligase complex subunits, enabling stabilization of protein targets such as HFR1 (Figure 2a and 2b).

The activity of COP1 is facilitated by the COP9 signalosome (CSN)

The COP9 signalosome (CSN) is a multi-protein complex consisting of eight subunits, six of which were identified in screens for *cop/det/fus* mutants. Thus, *Arabidopsis csn* mutants were first identified in *Arabidopsis* as photomorphogenic repressors of seedling development in the dark (Wei *et al.,* 1994). The CSN complex plays an important role in the regulation of the assembly/disassembly and activity of cullin-based E3 ligases. For this, the CSN directly interacts with cullin-containing E3 ubiquitin ligases and removes the ubiquitin-like modifier Nedd8/RUB1 from the cullin subunit (Lau and Deng 2012, Dong *et al*. 2015). Within the CSN, it is CSN5, a metalloprotease domain-containing subunit the one that displays deneddylase activity towards neddylated (or rubylated cullins). Cullin deneddylation triggers their association with CAND1 (Cullin-Associated Neddylation Dissociated 1), a 120 kDa protein, also known as TIP120 (TBP-Interacting Protein, 120 kDa), which binds only deneddylated cullins and promotes dissociation of the adaptor modules from the Cullin-Rbx1 subcomplex. This process is required for the release of the adaptors modules once their targets have been polyubiquitinated in order to search for new targets. Disassembly of adaptors modules from the CRLs complexes also avoids their self-ubiquitination once their function has been fulfilled (Liu *et al*., 2002; Oshikawa *et al*., 2003). Afterwards, cullin neddylation, by en enzymatic cascade similar to ubiquitination, facilitates dissociation of CAND1 and the assembly of a new target-loaded adaptor module. Therefore, continuous cycles of cullin neddylation/deneddylation ensure correct assembly of CRL E3 Ub ligases (including $CRL4^{COP1\text{-}SPA}$ complexes) and dynamic ubiquitination of a wide range of protein targets.

It has been shown that the CSN also regulates COP1 activity by controlling its subcellular distribution depending on the light conditions. Thus,under light conditions COP1 is more abundant in the cytoplasm. However, upon transition to darkness, COP1 accumulates in the nucleus in a CSN-dependent manner and targets positive regulators of photomorphogenesis (i.e. HY5) for degradation by the proteasome (Figure 1). Nuclear translocation of COP1 requires the N-terminal domain of CSN1, but it is uncertain the precise mechanism by which the CSN facilitates COP1 migration/accumulation to the nucleus (Wang *et al*., 2009).

The role of the COP10/DET1/DDB1 (CDD) complex in photomorphogenesis.

In addition to being a constituent of CRL4, DDB1 proteins also interact with CONSTITUTIVE PHOTOMORPHOGENIC10 (COP10) and DE-ETIOLATED1 (DET1) to form a stable complex termed CDD in plants (Schroeder *et al*., 2002; Yanagawa *et al*., 2004), which cooperates with CRL4 in response to developmental and stress signals, for example to promote degradation of transcription factor HY5 and DCAF protein DAMAGED-SPECIFIC DNA BINDING PROTEIN2 (DDB2) under dark conditions or upon DNA damage, respectively (Osterlund *et al*., 2000b; Castells *et al*., 2010). The CDD complex was originally isolated from floral meristems of cauliflower (a Brassica species related to Arabidopsis) using a biochemical purification procedure (Yanagawa *et al*., 2004). In this complex, DDB1 acts as the core adaptor and allows CRL4-CDD interaction (Chen *et al*., 2006; Bernhardt *et al*., 2010). COP10 is an E2 Ub conjugase variant (UEV) that lacks the catalytic cysteine required for Ub binding prior to target ubiquitination (Suzuki *et al*., 2002). Therefore, COP10 has no E2 activity, but instead it can enhance the activity of other canonical E2s, including those that associate with $CRL4^{COP1\text{-}SPA}$ complexes to repress photomorphogenesis. Indeed, it has been shown that COP10 associates in vivo with COP1 (Yanagawa *et al*., 2004; Lau and Deng, 2009). In agreement with its role in repression of light responses, *Arabidopsis cop10-4* weak mutants (note that all null *cop/det/fus* mutants are seedling lethal) show a de-etiolated phenotype, characterized by open cotyledons and short hypocotyls, when grown in the dark (Suzuki *et al*. 2002). In addition, *cop10-4* mutants display an early flowering phenotype under non-inductive short day (SD) conditions. Characterization of flowering defects in mutants of COP10 showed it represses gene expression of the florigen, *FT,* by controlling negatively and positively, respectively, the photoperiodic and autonomous flowering pathways (Kang *et al*., 2015*).*

Within the CDD complex, DET1 is a multifunctional protein with no recognizable domains that enables targeted degradation of specific regulatory proteins, such as HY5, HFR1 and DDB2. For this activity, DET1 may act as a substrate adaptor as it was shown that, together with COP10, DET1 is able to directly interact with HFR1 and facilitate its ubiquitination by CRL4 E3 ligases (Chen *et al*. 2006, Shi *et al*., 2015).. However, it has been proposed that DET1 activity requires recruitment of additional DCAF proteins (Lau and Deng, 2012). Whether COP1 is included in the list of DCAF proteins that associate with DET1 is a matter of controversy (Lau and Deng, 2012). Additionally, DET1 helps to stabilize PIF transcription factors in the dark (see below;

Dong *et al*., 2014). This mechanism should facilitate coordinated action of CDD together with PIFs to repress photomorphogenesis in darkness. Noteworthy, DET1 also acts in close association to chromatin to regulate gene expression. For this, DET1 binds preferably to non-acetylated tails of Histone H2B and interacts with transcription factors, such as circadian clock regulators CCA1 and LHY, becoming a transcriptional co-repressor of *TOC1* gene expression. Due to the pleiotropic phenotypes of *det1* mutants, it has been proposed that DET1, as part of CRL4-CDDD complexes, has a broader impact in gene expression beyond circadian clock function (Lau and Deng, 2012). Further details on DET1 function in the control of different plant biological processes are provided in the next section.

Interestingly, the CDD complex is conserved in humans where it has been termed DDD-E2 since it contains, in addition to DDB1 and DET1, a canonical E2 Ub conjugase (highly homologous to UEV COP10) and a small protein with no obvious motifs called DET1-, DDB1-Associated 1 (DDA1) (Pick *et al*., 2007). DDA1 is conserved in higher eukaryotes, including the model plant *Arabidopsis*. Functional characterization of human DDA1 (hDDA1) showed it acts as a positive regulator of CRL4s, likely in close association to chromatin, although the molecular basis of this activity remained unknown (Olma *et al*., 2009). Recently, a study from our laboratory demonstrated that DDA1 is a novel type of substrate receptor for CRL4-CDDD E3 ligases that recognizes and promotes ubiquitination and proteasomal degradation of specific protein targets (Irigoyen *et al*., 2014).

DET1 as a multifunctional protein controlling diverse plant processes.

As previously mentioned, DET1 acts as a repressor of photomorphogenesis by mediating targeted destabilization of different positive regulators of light signaling (i.e. HY5). More recently, it has been demonstrated that DET1 also plays a dual role in controlling the stability of both positive (HFR1) and negative (PIF1) regulators of seed germination in response to light,. Thus, it was found that DET1 enables PIF1 stabilization by inactivating an unknown protease. On the contrary, active DET1 recognizes and promotes degradation of HFR1 in the dark, inhibiting therefore seed germination (Dong *et al*., 2014; Shi *et al*. 2015). When seeds are exposed to light, DET1 is inactivated, resulting in increased HFR1 accumulation and decreased PIF1 levels. This contrasting effect on protein stability, together with HFR1-mediated inactivation of PIF1 by physical interaction, favors seed germination (Shi *et al*., 2015).

In addition to the afore-mentioned mechanisms, DET1-mediated stabilization and activation of PIFs likely involves repression of the DELLA proteins. The latter are negative regulators of gibberellins (GA) signaling that, in the absence of the hormone, mediate targeted degradation of PIFs (Davière and Achard 2016).. Light and gibberellins (GA) antagonistically regulate photomorphogenesis, with light repressing PIF function whereas GA helps to stabilize and activate them to promote cell elongation and plant growth. In this scenario, a recent study showed that DET1 function decreases the level of DELLA proteins in dark, enabling PIF accumulation and the promotion of a skotomorphogenic developmental pattern (Li *et al*., 2015).

DET1 not only represses photomorphogenesis but also represses flowering by altering the photoperiod and autonomous pathways. *det1-1* mutants are photoperiod-insensitive showing slightly early flower under LD and extremely early under SD conditions, whereas DET1 gain of function plants display late flowering, particularly under SD conditions (Kang *et al*., 2015). Flowering is controlled by an intricated regulatory network comprising several signaling pathways, termed photoperiod, autonomous, vernalization, thermosensory and gibberellin-dependent pathway. Within the photoperiod pathway, GI functions acts as a flowering inducer by activating *FT* transcription under flowering-inductive LD conditions. This process is negatively regulated by DET1, which interacts with GI and disrupts the interaction between GI and the *FT* promoter, decreasing therefore *FT* expression and delaying flowering. Thus, GI protein does not represent a target for ubiquitination and degradation by DET1, which means DET1 does not affect GI stability but functions as a repressor of *FT* transcription (Kang *et al*., 2015). In addition, DET1 may delay flowering by altering the transcription of floral repressor *FLOWERING LOCUS C* (*FLC*), which encodes a MADS-box transcription factor that blocks expression of both floral integrators FT and SOC1. For this, DET1 directly interacts with MULTICOPY SUPPRESSOR OF IRA1 4 (MSI4), which is part of a CUL4-DDB1 complex (CUL4-DDB1-MSI4). It was shown that CUL4-DDB1-MSI4 complexes associate with histone methyltransferase CLF-POLYCOMB REPRESSIVE COMPLEX (CLF-PCR2) and increase repressive H3K27 trimethylated marks on the *FLC* promoter. Therefore these complexes repress *FLC* gene expression and induce flowering. Accordingly, *det1* mutants exhibit altered FLC promoter methylation and expression (Pazhouhandeh *et al*., 2011, Kang *et al*., 2015). The current model proposes that, in addition to negative control of GI activity, DET1 contributes to the

upregulation of *FLC* expression, possibly by weakening the activity of MSI4 and CLF-PCR2 in histone modification of the *FLC* locus. These effects, in turn, should lead to reduced expression of *FT* and *SOC1* genes, and delayed floral transition.

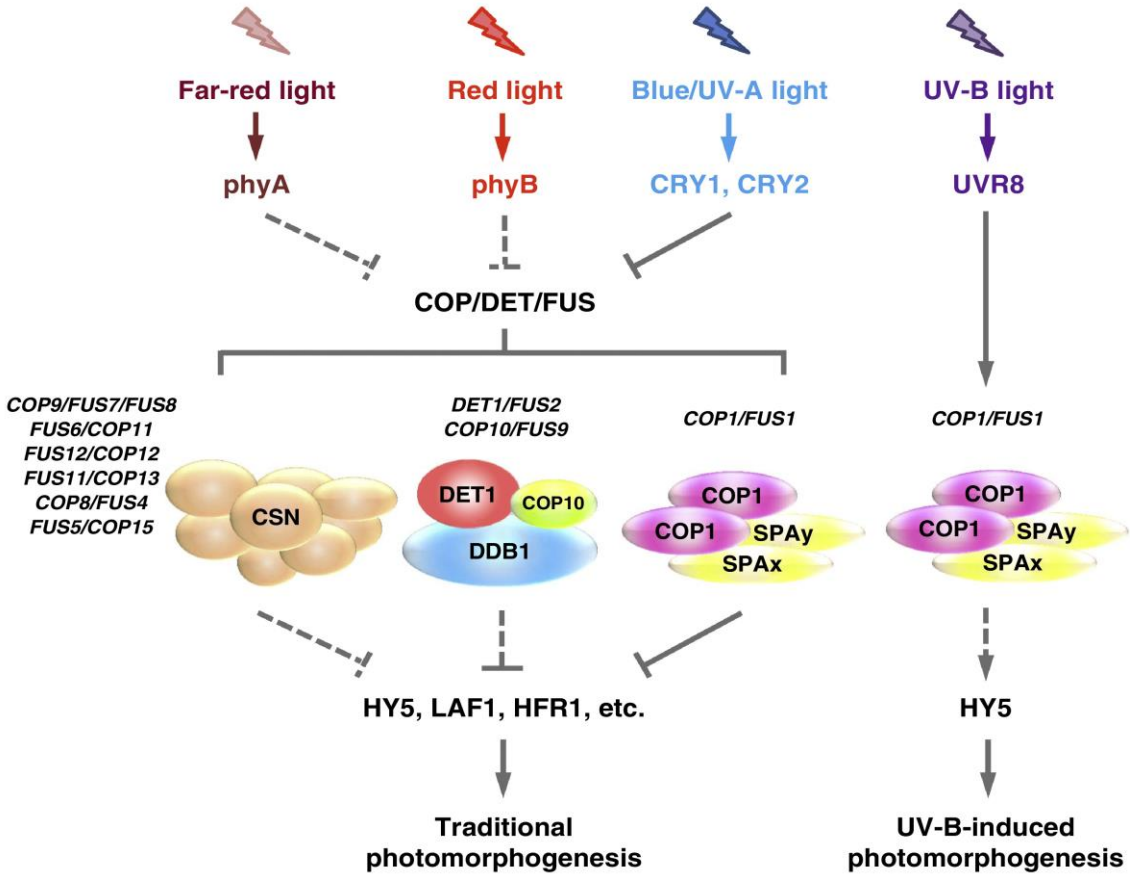


Figure 3. A simplified model of the involvement of COP/DET/FUS complexes in photomorphogenesis. Encoded by nine COP/DET/FUS genes, the COP/DET/ FUS proteins are components of the CSN complex, the CDD complex and the COP1–SPA complex. Photoreceptors are capable of sensing light ranging from UV-B to far-red light: phytochromes (phyA and phyB) for far-red and red light, cryptochromes (cry1 and cry2) for blue/UV-A light, and UVR8 for UV-B light. Within the conventional photomorphogenesis pathway, the COP/DET/FUS complexes repress light signaling by mediating the degradation of photomorphogenesis-promoting transcription regulators like HY5, LAF1 and HFR1. The activity of COP/DET/FUS complexes is inhibited by light signals. In UV-B-induced photomorphogenesis, the COP1–SPA complex accumulates and promotes light signaling by stabilizing HY5. The tetrameric COP1-SPA core complex consists of two COP1 proteins, and two SPA proteins. These two SPA proteins can be homogenous as indicated by SPAx, or heterogeneous as indicated by SPAx and SPAy. Note: solid lines represent direct effects, while dotted lines indicate indirect or unidentified direct regulation. Huang, *et al*. 2014.

Previous evidences had already pointed to a role of DET1 function in close association to chromatin. Thus, although it was known that *DET1* encoded a nuclear-localized no detectable DNA-binding activity could be found (Pepper *et al*., 1994). However, more than a decade ago,

Benvenuto *et al*., (2002) provided evidences supporting DET1 binding to the tail of non-acetylated H2B although the functional significance of this interaction and the molecular mechanisms underneath it remain a mystery (Benvenuto *et al*., 2002). Such a connection to chromatin was strengthened by recent studies connecting DET1 with circadian clock gene regulation. Thus, DET1 can interact with two MYB transcription factors, CCA1 and LHY and is recruited to their target genes. CCA1 and LHY are morning components of the circadian clock and repress evening genes as TOC1 (Lau et al, Moll cell 2011; review Lau and Deng,2012). CCA1 transcriptional repression activity of *TOC1* required DET1, indicating that DET1 plays an important role in transcriptional regulation, acting as a transcriptional co-repressor of the *Arabidopsis* circadian clock. Since DET1 associates to non-acetylated tails of H2B, DET1 might function as a transcriptional repressor in part by regulating chromatin conformation and accessibility to the transcriptional machinery, thereby affecting the expression of many genes. Additionally, it is reasonable to speculate that association of DET1 to chromatin might have evolved to 'hijack' the CUL4–DDB1 machinery to target interacting proteins or modulate the activity of other CUL4–DDB1 ligases in a chromatin context.

CRL4-CDDD-mediated control of ABA signaling.

Phytohormone ABA has a central role in the control of seed germination and the regulation of plant responses to abiotic stresses, such as drought, high salinity and low temperatures (Chinnusamy et al., 2008; Hirayama and Shinozaki, 2010; Hauser et al., 2011). ABA signaling is mediated by the pyrabactin resistance/pyrabactin resistance–like/regulatory components of ABA receptor (PYR/ PYL/RCAR) family of ABA receptors, which allows direct ABA-dependent inhibition of clade A phosphatases type 2C (PP2Cs), such as ABA INSENSITIVE1 (ABI1), HYPERSENSITIVE TO ABA1 (HAB1) and HAB2, and PP2CA, which are key negative regulators of the pathway (Saez *et al*., 2006; Rubio *et al*., 2009). Inhibition of PP2Cs leads to the activation of SUCROSE NONFERMENTING1–related subfamily 2 kinases that, in turn, regulate the transcriptional response to ABA by phosphorylating specific protein targets, including transcription factors of the ABA-responsive element binding/ABRE binding factor (ABF) family (Cutler *et al*., 2010; Nakashima and Yamaguchi-Shinozaki, 2013).

Targeted destabilization of key components of the ABA perception machinery, such as ABA receptors and PP2Cs, represents a key regulatory mechanism to modulate ABA signaling and,

therefore, plant adaptation to water deficiency stress. Previous work from our group showed that DDA1 acts as a substrate adaptor of CRL4-CDDD complexes (Irigoyen et al., 2014). Thus, by means of a yeast two hybrids screen using an Arabidopsis cDNA library, it was found that DDA1 binds to ABA receptor PYL8, as well as PYL4 and PYL9. Such interaction was confirmed in vivo and, moreover, it was shown that DDA1 facilitates PYL8 ubiquitination and proteasomal degradation. Accordingly, DDA1 acted as negative regulator of ABA-mediated developmental responses, including inhibition of seed germination, seedling establishment, and root growth. All other CDDD components displayed a similar regulatory function, although they did not directly interact with PYL8, further supporting the notion that, within the CRL4-CDDD complex, DDA1 displays substrate recognition activity. Interestingly, DDA1-mediated destabilization of PYL8 was counteracted by ABA, which protected PYL8 by limiting its polyubiquitination. These findings established a function for DDA1 as a substrate receptor for CRL4-CDD complexes and uncovered a mechanism for the desensitization of ABA signaling based on the regulation of ABA receptor stability (Irigoyen, *et al*. 2014).

DDA1 activity towards protein targets seems not to be limited to ABA receptors of the PYR/PYL/RCAR family since in the afore-mentioned yeast two hybrids screen additional protein, involved in different plant processes were found (unpublished data). These included, among others, transcription factors controlling cell division, dehydration stress proteins, kinases controlling energy metabolism, and a member of the chromatin remodeling machinery. The interaction of the latter with DDA1 and its functional relevance is the subject of the present study.

Chromatin remodeling and plant perception of light cues.

How chromatin remodeling, by means of histone modification, impacts plant responses to light, more particularly during seedling de-etiolation, has been the object of recent enlightening studies. When dark-grown seedlings break through the soil and reach the light for the first time, the seedlings switch to a photomorphogenic developmental pattern. Massive transcriptional changes underlie this developmental switch in response to light exposure, which are the result of coordinated activity of a set of transcription factors and chromatin remodeling machineries (Ma *et al.*, 2005; Lopez-Juez *et al.*, 2008). In this regard, two epigenomic studies that analyzed chromatin state variations in etiolated (dark-grown) and de-

etiolated (upon a shift to white light for 1 or 6 h) seedlings showed that transcriptional changes are significantly associated with local variations of specific histone post-translational modifications (Charron *et al.*, 2009; Bourbousse *et al.*, 2012). These studies not only defined precise chromatin changes along hundreds of genes but also provided insights on the mechanisms governing chromatin changes associated with light-driven developmental adaptations (described in Figure 4 and next sections).

Chromatin dynamics not only modulate seedling de-etiolation but also other types of light responses, such as the shade avoidance response (SAR) (van Zanten *et al.*, 2010). SAR is a developmental adaptation to abnormal light conditions, such as those encountered under the canopy, where plants receive light with a relative decrease in the red/far-red ratio, as red light is readily absorbed by the foliage at a higher rate than far-red light. A reduction in the red/far-red ratio is perceived by phyB, which triggers important modifications in several plant species including enhanced cell elongation, which leads to longer petioles at the rosette, smaller leaf blades, and suppressed axillary bud growth, to adapt to reduced light availability (Casal, 2013). Transcriptome analyses of *Arabidopsis* plants grown in the shade found that large changes in genome expression are triggered to promote SAR (Devlin *et al.*, 2003; Hornitschek *et al.*, 2012; Leivar *et al.*, 2012; Ciolfi *et al.*, 2013). By conducting a deep analysis of mutant plants altered in photoperception, histone modification, gene silencing, and transcriptional co-repression, a set of chromatin-based regulatory mechanisms impacting the regulation of light gene expression during SAR was identified (Casal, 2013).

Histone acetylation in controlling light signaling.

Histone acetylation is considered, in general, a positive activator of transcriptional activity. Addition of an acetyl group on histone lysine residues neutralizes the lysine positive charges and causes chromatin structure relaxation and greater accessibility for the transcriptional machinery (Lee and Workman, 2007). Acetylation can occur on multiple lysine residues on histone H3 (such as K9, 14, 18, 23, 27, and 56), histone H4 (K5, 8, 12, 16, and 20), and histone H2B isoforms (K6, 11, 27, 32, 38, 39, and 145 for HTB9) in *Arabidopsis* (Johnson *et al.*, 2004; Bergmuller *et al.*, 2007; Earley *et al.*, 2007; Zhang *et al.*, 2007*a*). Precise regulation of histone acetylation is important for the control of many plant biological processes, including photomorphogenesis. For this, as described below, various classes of histone

acetyltransferases (HATs) and histone deacetylases (HDACs) have been described in plants (Lee and Workman, 2007; Shahbazian and Grunstein, 2007).

Several studies in *Arabidopsis* and other plant species have shown that changes in the expression of various light-responsive genes correlate with histone H3 and H4 acetylation modifications. Thus, by comparing H3K9ac levels in dark- and light-grown seeedlings, it was found that the levels of this epigenetic mark correlates with the light intensity and a rise in mRNA levels at several light-responsive genes (Guo *et al.*, 2008). This is the case of *CHLOROPHYLL A/B-BINDING PROTEIN 2* (*CAB*) and *LIGHT HARVESTING CHLOROPHYLL A/B-BINDING PROTEIN 1.1* (*LHCB1.1*) that in response to different light wavelengths increased their expression as well as they accumulate histone acetylation marks at their promoter regions (Bertrand *et al.*, 2005; Benhamed *et al.*, 2006; Offermann *et al.*, 2006; Casati *et al.*, 2008; Liu *et al.*, 2013). Similarly, increased *PHYA* expression upon dark-to-light shifts is joined by increased H3 acetylation at K9/K14 and K27 and histone H4 hyperacetylation (Jang *et al.*, 2011). The relevance of histone acetylation in plant responses to light was further demonstrated by a genome-wide survey of histone H3 acetylation changes at K9 and K27 during de-etiolation (Charron *et al.*, 2009). This study revealed rapid and massive variations of acetylated domains at light-regulated genes for these two marks.

Additional evidence on the role of histone acetylation in light signaling comes from the study of *Arabidopsis* mutants defective in histone acetylation (HAT) and deacetylation (HDAC) activities, which in both cases showed light insensitive phenotypes. For instance, loss of function of the HAT activity of TATA-BINDING PROTEIN ASSOCIATED FACTOR1 (TAF1) resulted in decreased acetylated levels of H3 in the *CAB2* gene promoter that correlated with reduced *CAB2* transcription (Benhamed *et al.*, 2008). Furthermore, mutation in *GENERAL CONTROL NON-REPRESSIBLE5* (*GCN5*), a core subunit of the histone acetylase module within the SAGA complex, resulted in a long-hypocotyl phenotype and reduced light-inducible gene expression that correlated with a reduction on acetylated histones H3 and H4 (notably K14/K27 and K8/K12, respectively) on their promoter regions (Benhamed *et al.*, 2006). Oppositely to HATs, *Arabidopsis* HDACs reduce the acetylation levels on histone H3 and H4 on light-regulated genes. This is the case of HISTONE DEACETYLASE 1/19 (HD1/HDA19), which represses the acetylation in H3K9, H3K27, H4K5 and H4k8 residues of light-responsive genes, including *RBCS-1A*, *CAB2*, *LHCB1.4* and *PHYA*, reducing their expression (Benhamed *et al.*, 2006; Guo *et al.*,

2008; Jang *et al.*, 2011). On the contrary, *Arabidopsis* mutation of *HDA15* exhibited a long hypocotyl in far-red and red light, indicating that this HDAC plays a role as a positive regulator of light development. Interestingly, HDA15 was found to physically associate with PIF3 and to be required for PIF3-binding to promoters of target genes. This process seemed to be reversible and controlled by light conditions, as HDA15-PIF3 association at specific target genes in etiolated seedlings was impaired upon exposure to red light (Liu *et al.*, 2013).

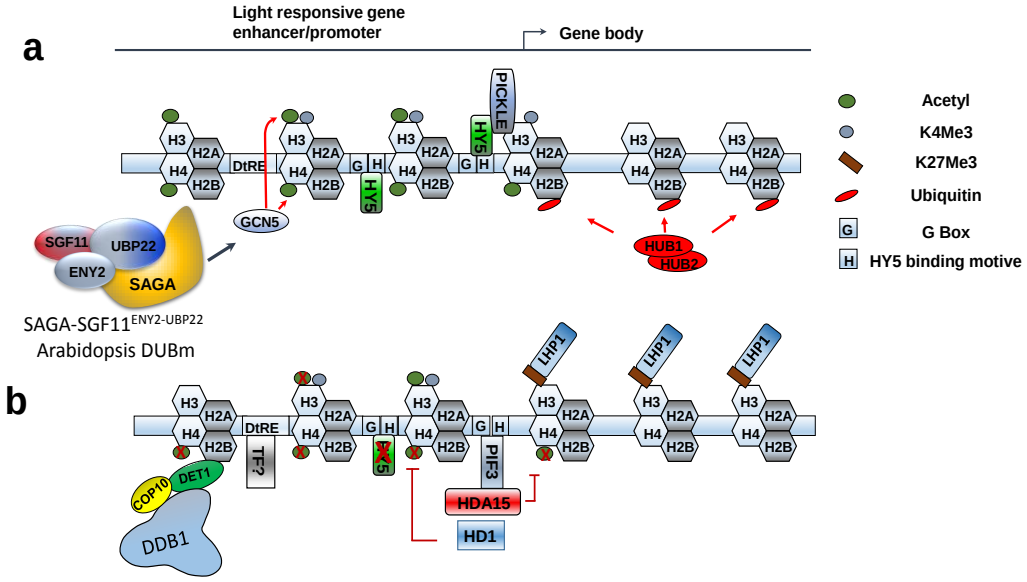


Figure 4. Schematic model depicting histone marks and chromatin-associated factors on light-responsive genes. (**a**) Active states. Transcription initiation of light-responsive genes is impacted with acetylation of histone H3 and H4 at various lysine residues on 5' domains and by H3K4me3 deposition around the transcription start site. Monoubiquitination of histone H2B can facilitate RNA Pol II elongation along the transcribed region. HY5 is an essential transcription factor downstream from photomorphogenesis that can bind DNA in light and dark conditions with opposite effects on transcription in different contexts. GCN5 is a major histone acetyltransferase in this process that may be recruited on light-responsive genes through a HY5-dependent pathway. The PICKLE chromatin remodeller may also modulate gene expression when recruited by HY5, possibly as an activator or as a repressor. (**b**) Repressed states. Histone deacetylation through HD1, HDA15, or other undetermined histone deacetylases is required for gene repression. PIF3 may recruit HDA15 around G-box motifs at light-inducible promoters to repress transcription in darkness. PIF3 may also have an opposite effect for transcriptional activation in different contexts through unknown mechanisms. Unknown transcription factors (TF) possibly allow the recruitment of DET1 to light-regulatory DNA sequences such as DET1-Dark Response Elements (DtREs). Coincident chromatin deacetylation may increase the affinity of DET1 for histone H2B. Chromatin-bound DET1 promotes strong transcriptional repression through a mechanism that presumably involves HY5 proteolysis. LHP1 may contribute to the maintenance of H3K27me3-containing repressive states. Ovals indicate factors and histone modifications that associate with expressed genes, and rectangles indicate transcriptional repressors. Dashed lines indicate predicted interactions. (adapted from Barnache, *et al.* (2014)).

It has been shown that the role of histone acetylation in the control of light-responsive genes also occurs in other plant species. Thus, in green shoots of tobacco plants, histones H3 and H4

on the *PetE* promoter were hyperacetylated, suggesting that histone acetylation facilitates light-responsive gene transcription to trigger a developmental switch in tobacco (Chua *et al*., 2001, 2003).

SAGA complex in the context of chromatin remodeling.

Eukaryotic transcriptional coactivators are multi-protein complexes associated with histone modification and chromatin remodeling that help to recruit the transcriptional machinery at specific gene promoters (Näär *et al*., 2001). SAGA (Spt–Ada–Gcn5 Acetyltransferase) is a transcriptional coactivator complex involved in the regulation of numerous cellular processes through the coordination of the post-translational modification of various histones. The yeast SAGA complex is involved in histone acetylation (HAT)(Grant *et al*., 1997), histone deubiquitination (Daniel *et al*., 2004), mRNA export (Rodríguez-Navarro *et al*., 2004), transcription elongation (Govind *et al*., 2007), chromatin recognition (Pray-Grant *et al*., 2005), and regulation of the basal transcriptional machinery (Sterner *et al*., 1999). Unraveling the modular composition of the SAGA complex has enabled interpretation of its multifunctional role (Wu *et al*., 2004), principally in regulating the transcription of many stress-inducible and developmentally regulated genes (Huisinga and Pugh, 2004; Wang and Dent, 2014). The diverse functions of SAGA involve the participation of modules that are highly conserved between yeast, flies, and mammals. The SAGA complex is composed of more than 21 subunits, that are broadly conserved from yeast to humans, arranged in four modules: a histone acetyltransferase module, the deubiquitinating module, and the SPT and TAF modules, which are implicated in the complex assembly, chromatin recruitment and SAGA architecture (Daniel and Grant, 2007; Koutelou *et al*., 2010). However, little knowledge is available about the composition and functions of the plant SAGA complex. A recent study identified candidate genes encoding subunits of the SAGA complex in some plants species (Srivastava *et al*., 2015), suggesting conservation of the SAGA complex throughout evolution.

The involvement of the SAGA complex in the control of light responses in plants comes mainly from the functional characterization of its GCN5 subunit. Thus, as afore-mentioned, *Arabidopsis gcn5* mutants showed long hypocotyls in response to light compared to wild type controls, indicating that GCN5 acts as a positive regulator of photomorphogenesis (Benhamed *et al.*, 2006). (Figure. 4).

Although first characterized as a histone acetyltransferase, because of the GCN5 subunit, the SAGA complex is known to contain a second activity in other model organisms, such as yeast, fly and humans. This activity corresponds to a histone deubiquitinase (Koutelou *et al*., 2010) that remains to be characterized in plants (Feng and Sheng 2014). The SAGA deubiquitination module (DUBm) in yeast and humans is composed by 4 proteins: UBIQUITIN PROTEASE8 (Ubp8)/UBIQUITIN-SPECIFIC PEPTIDASE22 (USP22), SAGA-ASSOCIATED FACTOR11 (Sgf11)/ATAXIN-LIKE (ATXN7L3), (Sus1)/ENHANCER OF YELLOW2 (ENY2) and Sgf73/ATAXIN7 (ATXN7), respectively (Rodriguez-Navarro, 2009). Null mutations in the yeast DUBm components do not affect their viability. However, mutations in components of the SAGA DUBm or altered expression of SAGA subunits correlate with neurological disease and aggressive cancers in humans, highlighting the functional relevance of this activity in higher eukaryotes (Weake *et al*., 2008, Zhang *et al*., 2008). In Drosophila, although a DUBm composed by just three subunits (a single protein might acts as SGF11 and SGF73 together) has been described, its association to the rest of the SAGA complex has not been proven. It has been recently shown that Drosophila SGF11 protein is related to mRNA export by associating with a component of the cap-binding complex and with the mRNA export complex (AMEX) (Gurskiy *et al*., 2012). Additionally, Drosophyla e(y)2, the ortholog of ENY2, seems to have a gonad-specific function (Krasnov *et al*., 2005).

Histone ubiquitination and transcriptional activation.

Modification of histones by mono ubiquitination seems to be limited to both H2A and H2B in eukaryotic organisms. Thus, H2A is mono-ubiquitinated at Lys-119 (H2Aub) in higher eukaryotes (Bohm, *et al*. 1980, Swerdlow, *et al*. 1990) whereas H2B is mono-ubiquitinated at Lys-120 (ubH2B) in mouse cells, Lys-123 in *Saccharomyces cerevisiae*, Lys-119 in *S. pombe*, and Lys-143 in *Arabidopsis.* Histone H2B monoubiquitination (H2Bub) is part of a general mechanism that influences transcriptional activity positively (Weake and Workman, 2008). Thus, H2Bub was found to facilitate the recruitment or RNA Pol II to the genes promoter where the Facilitates Chromatin Transcription (FACT) machinery associates, in order to facilitate transcription initation, and to ensure nucleosome reassembly upon RNA Pol II elongation (Belotserkovskaya *et al.*, 2003; Pavri *et al.*, 2006; Fleming *et al.*, 2008; Xin *et al.*, 2009; Fierz *et al.*, 2011).

According to its role in promoting transcriptional activity, H2B ubiquitination is highly a dynamic and reversible process where both ubiquitination and deubiquitination participate to tightly control H2Bub levels at specific loci. In *S. cerevisiae,* E2 ubiquitin-conjugating enzyme, *Rad6*, in association with E3 ubiquitin ligase *Bre1*, catalyzes monoubiquitylation of histone H2B. In *Arabidopsis* H2B is ubiquitinated on lysine 143 (Bergmuller *et al.*, 2007) by the heterodimeric of E3 ubiquitin-protein ligase HISTONE MONO-UBIQUITINATION 1/2 (HUB1/ HUB2) Bre1 homologue (Fleury *et al.*, 2007; Cao *et al.*, 2008; Gu *et al.*, 2009; Xu *et al.*, 2009). HUB1/2 activity results in H2Bub mark distribution along the promoter regions within gene bodies but not on promoters in the *Arabidopsis* genome (Roudier *et al.*, 2011), in accordance with its regulatory role in transcription elongation in various organisms (Shilatifard, 2006; Berger, 2007). Characterization of *Arabidopsis hub1/2* mutants showed almost undetectable H2Bub levels. In addition, this mutants displayed reduced levels of H3K4me3 mark in the coding region of *FLOWERING LOCUS C (FLC)* which likely caused the early flowering phenotype in these mutants (Cao, *et al.*, 2008, Sun and Allis, 2002). In addition, it has been shown that HUB1 function influences the expression of dormancy related genes (Liu *et al*., 2007). H2Bub was also shown to be required to reach maximal transcript levels of Circadian clock genes in *Arabidopsis* (Himanen *et al*., 2012). With regard to photomorphogenesis, a wide genomic approach showed that H2B monoubiquitination mark allows accurate and dynamic gene expression of light-responsive genes (Bourbousse *et al*., 2012) Thus, a correlation between H2Bub mark enrichment and gene upregulation was found, whereas in *hub1* mutants that lack H2Bub, a large number of light responsive genes lost their rapid response to light, meaning that H2Bub is important for attaining appropriate expression levels (Bourbousse at al., 2012).

From these studies, it can be deducted that H2B ubiquitination is highly a dynamic and reversible process. Removal of ubiquitin from H2Bub is catalyzed by ubiquitin specific proteases or deubiquitinating enzymes (DUBs), such as UBPs in yeast and plants, and USPs in mammals, including the SAGA DUBm (Hicke, 2001; Zhang, 2003; Smalle and Vierstra, 2004). Whereas the role of the SAGA DUbm in H2B deubiquitination has been characterized in detail in yeast and mammals, the function of DUBm in plant still poorly understood. In this study, we functionally characterize for the first time the Arabidopsis DUBm, demonstrating its involvement in H2Bub deubiquitination, and unveil a new role for this complex in facilitating CRL4-CDDD activity as a repressor of *Arabidopsis* photomorphogenesis regulation. In this way, we propose a molecular mechanism by which targeted destabilization of positive regulators of

light signaling is coordinated with chromatin modification to repress gene expression in response to light.

OBJECTIVES

Objectives of the work.

Prior to this study there was no evidence regarding DUBm function in plants. The fact that SGF11 interacted with DDA1, a component of CRL4-CDDD E3 ligases, suggested a potential link between the chromatin remodelling machinery and the ubiquitin proteasome system which could have an impact in plant growth and development under changing light conditions. This idea led us to investigate the functional and physiological relevance of the DUBm/CRL4-CDDD interaction. With this aim, we proposed the following specific objectives:

1. Biochemical characterization of the DUBm complex and its association with CRL4-CDDD E3 ubiquitin ligase.
2. Functional characterization of the Arabidopsis DUBm complex.
3. Study of the effect of CRL4-CDDD E3 ligases on the stability of DUBm components and in ubiquitinated H2B (H2Bub) abundance.
4. Characterization of the effect of the DUBm on CRL4-CDDD-mediated control of photomorphogenesis.

MATERIALS AND METHODS

Materials and Methods

Biological materials.

Bacterial strains.

- *Agrobacterium tumefaciens* (*A. tumefaciens*) C58C1Rif^{R} (pGV2260) (Deblaere *et al*., 1985).

- *A. tumefaciens* C58C1Rif^{R} (pMP90) (Koncz, 1986).

- *Escherichia coli* (*E. coli*) DH5α (Woodcock *et al*., 1989).

Yeast strain.

- *Saccharomyces cerevisiae* (*S. cerevisiae*) AH109 (Clontech).

- Yeast strain 120

Plant material.

- *Arabidopsis thaliana* (Arabidopsis) (L.) Heynh. ecotype Columbia-0.

- *Nicotiana benthamiana* (*N. benthamiana*).

- Loss of function mutants in Arabidopsis Columbia-0 genetic background (Table 1).

- Stable transgenic Arabidopsis lines in Columbia-0 genetic background (Table 2).

- PSB-D cell suspensions of Arabidopsis (L.) Heynh. ecotype Landsberg erecta (Ler), derived from MM2d (Menges and Murray 2002).

Table 1. Mutant alleles and *Col-0* transgenic lines.

Stable line	Ref	Observation	TAG
Sgf11-1	This study	SAIL-856-F11	
Sgf11-2	This study	SALk_090794c	
Ubp22-1	This study	GK-263H06	
det1-1	Pepper *et al*., 1994		
cop1-4	Yu *et al*., 2008		
hub2-2	Liu *et al*., 2007		
hy5-215	Oyama *et al*., 1997		
OeMYC-SGF11	This study		MYC
OeGFP-SGF11	This study		GFP
OeDDA1-GFP	Irigoyen *et al*., 2014		GFP
OeMYC-ENY2	This study		MYC
OeGFP-ENY2	This study		GFP
OeGFP-UBP22		Gift from F. Barneche	GFP
OeGFP-DET1	Schroeder *et al*., 2002		GFP
Sgf11-1/det1-1	This study		
OeMYC-SGF11/*det1-1*	This study		
Sgf11-1 / OeGFP-DET1	This study		
ubp22-1/ *det1-1*	This study		

Cloning procedures.

Most of the plasmid constructs were generated using the gateway technology (Life technologies), following the manufacturer recommendations. Desired genes were amplified by PCR (Sambrook *et al*., 1989)using the enzyme "Pwo DNA polymerase" (Roche) and specific primers carrying the flanking regions recognized by bacteriophage λ: attB1 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATACAAA-(insert)-3') and *attB2* (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTA-(insert)-3') (Table 2). PCR products were separated by gel electrophoresis and purified with the "QIAquick® Gel extraction kit" (Quiagen) and inserted into pDONR207 gentamicin^{R} vector or into pDONR221 kanamicin^{R} vector (Table 2) using the enzyme "Gateway® BP Clonase™ II Enzyme Mix" (Life Technologies) and following the manufacturer recomendations. The generated plasmid constructs were transformed into *E. coli* DH5α by the heat-shock method (Sambrook *et al*., 1989), and grown in Luria-Bertani (LB) media, supplemented with the adequate antibiotics. Plasmid were isolated by miniprep

extraction using the "QIAprep® Mini-Prep kit" and analysed by analytical digestion and sequencing.

Table 2. $pDONR_{207}$, $pDONR_{221}$ and pENTR™ constructs.

Construct	Plasmid	Selection marker	Observation	Cloning tec.
SGF11	$pDONR_{207}$	Gentamycin		Gateway
SGF11-STOP	$pDONR_{207}$	Gentamycin		Gateway
SGF11	$pDONR_{221}$	Kanamycin		Gateway
PSGF11	$pDONR_{207}$	Gentamycin		Gateway
UBP22	$pDONR_{207}$	Gentamycin		Gateway
ENY2	$pDONR_{207}$	Gentamycin		Gateway
DET1	$pDONR_{207}$	Gentamycin	Irigoyen *et al*., 2014	
COP10	$pDONR_{207}$	Gentamycin	Irigoyen *et al*., 2014	
COP1	$pDONR_{207}$	Gentamycin	Irigoyen *et al*., 2014	
DDA1	pDONR207	Gentamycin	Irigoyen *et al*., 2014	
DDB1A	pDONR207	Gentamycin	Irigoyen *et al*., 2014	
DDB1B	pDONR207	Gentamycin	Irigoyen *et al*., 2014	
pEN-L4-2-L3	$pENTR^{TM}$	Kanamycin	http://gateway.psb.ugent.be	Gateway
pKNGS_rhino	$pENTR^{TM}$	Spectinomycin	http://gateway.psb.ugent.be	Gateway

Table 3. Primers used for cloning fragments amplification for gateway constructs.

No.	Insertion	Primer sequence	observation
1	SGF11 BF	GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGTCTGGCGCAGAGGATAAT	pDONR
2	SGF11 BR	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAGTCTCCTTTCACGTTCTCTCG	pDONR
3	SGF11BR- stop	GGGGACCACTTTGTACAAGAAAGCTGGGTATTCTCCTTTCACGTTCTCTCG	pDONR
4	ENY2 BF	GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGAAACATTCGGTGAATCG	pDONR
5	ENY2 BR- stop	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAAAACACCATCAAAGAGC	pDONR
6	UBP22 BF	GGGGACAAGTTTGTACAAAAAAGCAGGCTATATGTCCGCGATTTCATTTC	pDONR
7	UBP22 BR- stop	GGGGACCACTTTGTACAAGAAAGCTGGGTAGCAATCAGCAAAGGGAAATGC	pDONR
8	pSGF11 BF	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTCACCACAGAAGAAAAGAAG	pDONR
9	pSGF11 BR	GGGGACCACTTTGTACAAGAAAGCTGGGTACATTGGAATCCTCTAAATACGAG	pDONR

Generation of binary constructs for expression in plants.

Sequences inserted in entry vector indicated in table 2 were transferred to the final binary plasmids indicated in table 4 by the "Gateway LR Clonase™ II" (Life Technologies), following the manufacturer recommendations. The generated plasmid constructs were transformed into *E. coli* DH5α, isolated by miniprep extraction using the "QIAprep® Mini-Prep kit" and analysed

by analytical digestion and sequencing. The pGWB3, pGWB5 pGWB6 and pGWB21(Nakagawa *et al*., 2007) were used for fusion to GUS, GFP, and the MYC tags.

Table 4. Binary constructs used for expression in plants.

Construct	Plasmid	Selection marker	Observation	Cloning tec.
35S::MYC-SGF11	pGWB21	Kanamycin		Gateway
35S::GFP-SGF11	pGWB6	Kanamycin		Gateway
35S::DDA1-GFP	pGWB5	Kanamycin	Irigoyen *et al*., 2014	
35S::MYC-ENY2	pGWB21	Kanamycin		Gateway
35S::GFP-ENY2	pGWB6	Kanamycin		Gateway
***35S::*GFP-UBP22**	pGWB6	Kanamycin	Fredy Barneche.	Gateway

Generation of Bimolecular Fluorescent Complementation (BiFC) constructs.

To identify protein–protein interactions *in vivo* using Bimolecular Complementation Fluorescent (BiFC) technique desired genes were fused to the N-terminal or C-terminal E-YFP (yellow fluorescent protein) from Clontech (Table 5).

Table 5. Bimolecular Complementation Fluorescent (BiFC) constructs.

Construct	Selection marker	Observation	Cloning
pBIFC1			
DDA1: nYFP	Spectinomycin	Irigoyen *et al*., 2014	Gateway
pBIFC2			
nYFP:SGF11	Spectinomycin		Gateway
nYFP:ENY2	Spectinomycin		Gateway
nYFP:UBP22	Spectinomycin		Gateway
nYFP:DET1	Spectinomycin	Irigoyen *et al*., 2014	Gateway
nYFP:COP10	Spectinomycin	Irigoyen *et al*., 2014	Gateway
pBIFC3			
cYFP:SGF11	Spectinomycin		Gateway
cYFP:ENY2	Spectinomycin		Gateway
cYFP:UBP22	Spectinomycin		Gateway
cYFP:DET1	Spectinomycin	Irigoyen *et al*., 2014	Gateway
cYFP:COP10	Spectinomycin	Irigoyen *et al*., 2014	Gateway
pBIFC4			
DDA1:cYFP	Spectinomycin	Irigoyen *et al*., 2014	Gateway

All Fragments were generated under the control of 35S promoter. There are four plasmids were used for pBIFC and their structure map were as follow:

-pBIFC1: Gateway Site + Fragment N-terminal of nYFP.

-pBIFC2: Fragment N-terminal of YFP + Gateway Site.

-pBIFC3: Fragment C-terminal of YFP + Gateway Site.

-pBIFC4: Gateway Site + Fragment C-terminal of YFP

Generation of Yeast two hybrid constructs.

For yeast two hybrid studies, modified *Gateway* versions of pGADT7 and pGBKT7 vectors (Clontech) were used. These plasmids were generated by insertion of the *Nde*I-*Xho*I Gateway *ccdB* cassette into the *Nde*I-*Sal*I sites of the vector multiple cloning site (MCS) (assigned by Dr. Marta Boter, CNB, CSIC). Desired genes were inserted into these vectors by incubation with the LR Clonase™ (as mentioned before) and were expressed fused to the GAL4 activation domain (AD) or GAL4 binding domain (BD) when pGADT7 or pGBKT7 were used (Table 6).

Table 6. Yeast two hybrid constructs.

Construct	Selection marker	Observation	Cloning technique
pGBKT7 (GAL4 binding domain, bait)			
SGF11	Kanamycin		Gateway
ENY2	Kanamycin		Gateway
UBP22	Kanamycin		Gateway
DDA1	Kanamycin	Irigoyen *et al*., 2014	Gateway
DDB1a	Kanamycin	Irigoyen *et al*., 2014	Gateway
DDB1b	Kanamycin	Irigoyen *et al*., 2014	Gateway
DET1	Kanamycin	Irigoyen *et al*., 2014	Gateway
COP10	Kanamycin	Irigoyen *et al*., 2014	Gateway
pGADT7 (GAL4 activation domain, prey)			
SGF11	Ampicilin		Gateway
ENY2	Ampicilin		Gateway
UBP22	Ampicilin		Gateway
DDA1	Ampicilin	Irigoyen *et al*., 2014	Gateway
DDB1a	Ampicilin	Irigoyen *et al*., 2014	Gateway
DDB1b	Ampicilin	Irigoyen *et al*., 2014	Gateway
DET1	Ampicilin	Irigoyen *et al*., 2014	Gateway
COP10	Ampicilin	Irigoyen *et al*., 2014	Gateway

Cloning yeast genes for complementation in yeast.

Standard molecular biology techniques were used. Plasmids and yeast strains used in this study are described in Table 7. Yeast deletion alleles were received them from the Fredy Barnache lab, followed by transformation into yeast cells using standard yeast transformation techniques (http://www.fhcrc.org/labs /gottschling). We cloned the full-length UBP22 CDS in a Gateway vector into the individual strain backgrounds. One-step gene replacement was performed by transformation using standard techniques (Katan-Khaykovich and Struhl. 2002).

Table 7. Yeast strain and plasmids.

Plasmid, strain, or oligonucleotide	**Genotype or description**	**source**
-Plasmid		Fredy Barneche
pRG422	1.4-kb BamHI-SacI *FLAG-HTB1* fragment from pRS314-FLAG/HTB1 used to replace the corresponding region in pJH23	Fredy Barneche
-Yeast strain AR120	*HMLa MAT*a *HMR*a *cdc7-1 bar1 trp1-289 ura3-52 leu2-3,112 his6*	Fredy Barneche
UCC6389	MATa lys2D0 trp1D63 his3D200 ade2D::hisG ura3D0 leu2D0 met15D0 htaI-htb1::MET15 hta2-htb2::LEU2 ADE2-TEL-VR URA3-TEL-VIIL pRG422	Fredy Barneche
UCC6390	UCC6389 *ubp10_*::*NatMX*	Fredy Barneche
UCC6392	UCC6389 *ubp8_*::*KanMX*	Fredy Barneche
UCC6396	UCC6394 *sir4_*::*KanMX*	Fredy Barneche

Generation of expression vectors in Arabidopsis cell cultures line (PSB-D).

For expression of proteins fused to the TAP epitope in Arabidopsis cell cultures PSB-D (ABRC clone no. CCL84840) we used pDONR221:SG11 as input vector and the "Standard MultiSite Gateway LR reaction" technology to generate the plasmids 35S::Gsrhino-SGF11, carrying the TAP fusion at the N-terminus of SGF11. Double affinity tag were fused to SGF11 in 35S::GSrhino-SGF11, which consists in two repeats of the G protein, a cleavage site of

rhinovirus 3C protease and a binding site to streptavidin ("Streptavidin Binding Peptide", SBP). For the generation of these vectors the following combinations, as is described in Van Leene *et al*., 2015 and in figure 5, were used:

35S::Gsrhino-SGF11: pEN-L4-2-L3 (with 35S promotor) + pKNGS_rhino (Gs TAP tag) + pDONR221:SGF11

All vectors used are available in http://gateway.psb.ugent.be.

Plasmids were transformed into *E. coli* DH5α and selected for resistance to spectinomycin. Bacteria with the plasmid of interest were extracted with the kit "Mini-Prep kit QIAprep®" (Qiagen), and analyzed by analytical digestion and sequencing. Plasmids were subsequently transformed into *A. tumefaciens* C58C1 strains pMP90, and the colonies of interest were selected by their resistance to spectinomycin and gentamycin.

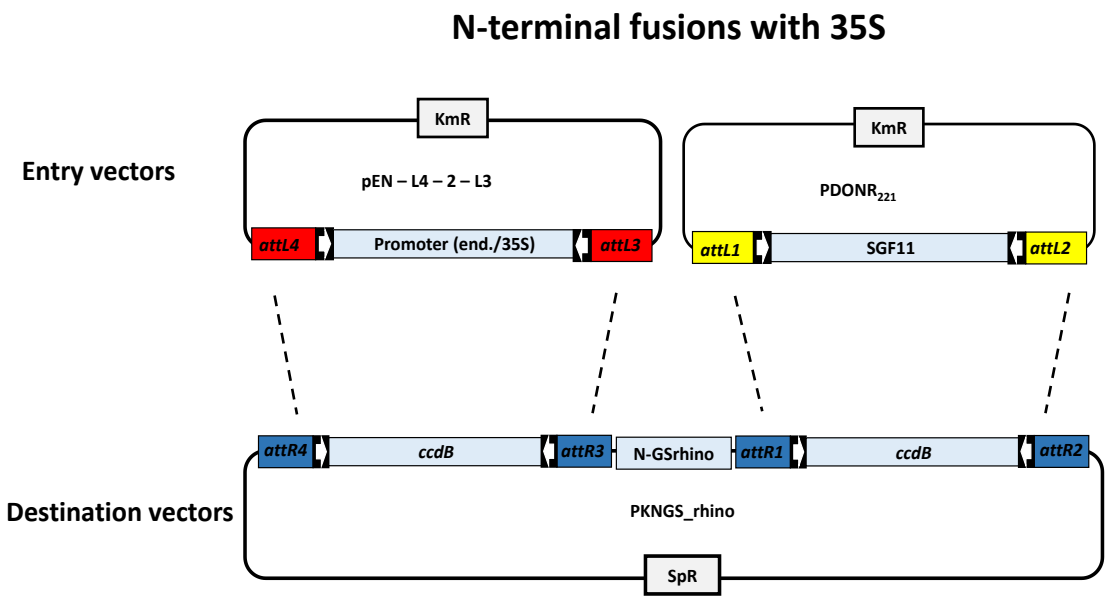


Figure 5: Cloning strategy of construction TAP tag scheme. 35S promoter Cauliflower; ccdB: toxic gene for negative selection of not recombinant plasmids; Km^R: gene kanamycin resistance; attL and attR: left and right edges of the T-DNA. Adapted from Van Leene *et al*., 2008.

Culture methods.

Bacterial culture methods.

Lysogeny broth (LB) medium was used for all the bacterial cultures (10 g/L Tryptone, 5g/L yeast extract and 10 g/L NaCl). pH was adjusted to 7.0 with NaOH. 15 g/L bactoagar were added for

solid media (Sambrook *et al*., 1989). *E. coli* and *A. tumefaciens* were cultured at 37 ºC and 28 ºC respectively with agitation at 250 r.p.m 30 min. Media were supplemented with the corresponding antibiotics: ampicilin (100 μg/mL), gentamicin (50 μg/mL), hygromycin (40 μg/mL), kanamycin (50 μg/mL), rifampicin (50 μg/mL), spectinomycin (50 μg/mL), streptomycin (10 μg/mL) and tetracycline (5 μg/mL).

Yeast two hybrid culture.

AH109 *S. cerevisae* cells were grown in YPAD and YPD media, respectively. Both media have almost the same composition (20 g/L peptone/tryptone, 10 g/L yeast extract, 40 % glucose, 40 mg/L adenine only in YPAD medium, pH adjusted to 5.8 with HCl 37 % and 20 g/L bactoagar added only for solid media) (Clontech Yeast Protocols Handbook). Transformation of pGADT7 and pGBKT7 led to normal growth of AH109/Y187 yeast cells on the selective media SD-WL. Cells were selected in YSD-WL medium for the presence of both pGADT7 and PGBKT7 plasmids, and overnight cell cultures grown in this selection media plated in the auxotrophic YSD-WLH and YSD-WLHA media to test for interactions. Yeast growth and handling was conducted according to the "Yeast Protocols Handbook" using the medium:

YSD-WL: SD Minimal Agar Base (Clontech) supplemented with the -Leu/-Trp DO Supplement (Clontech).

YSD-WLA: SD Minimal Agar Base supplemented with the -Ade/-Leu/-Trp DO Supplement (Clontech) and 0.003% adenine.

YSD-WLH: SD Minimal Agar Base supplemented with the -Leu/-Trp/-His DO Supplement (Clontech) and 0.003% adenine.

YSD-WLHA: SD Minimal Agar Base supplemented with the -Leu/-Trp/-His/-Ade DO Supplement (Clontech).

Different concentrations of 3-amino-1,2,4-triazole (3AT) were added in the media without histidine (YSD-WLH and YSD-WLHA), as an extra addition factor for colony selection, which is an inhibitor of the gene product HIS3, involved in histidine biosynthesis in *S. cerevisiae*. The growth temperature was 30 ° C and agitation in case of liquid medium was 250 rpm.

Complentation yeast culture.

Preparation of cell extracts and Western blot analysis — For UPB22 complentation of Ubp8 deletion, cultures were grown to exponential phase (OD600 from 0.2 to 0.6) in YP medium (1% bacto-yeast extract and 2% bacto-peptone) containing 2% glucose. Whole cell extracts (approximately 1x108 cells) were prepared by vortexing cells with glass beads 7 times (30 sec/time) in cold lysis buffer〔50 mM Tris-HCl, pH 7.5, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 0.5% Triton X-100, 10% glycerol, 1 mM DTT, complemented with PMSF (1 mM), and complete protease inhibitor mixture (Roche). whole-cell extracts were obtained using an urea/SDS buffer. Then, about 20µg of proteins were loaded on a 14% Tris-Tricine gel and H2B/H2Bub were detected using an anti-Flag antibody.

Plant cultivation.

To prepare plants for in vitro cultivation, Arabidopsis seeds were sterilized by sodium hypochlorite 75% v/v and 0.01% Tween-20 for 7 minutes with agitation, and washed up to 5 times with distilled water (milli-Q H2O). Seeds were plated in the germination media "Murashige & Skoog" containing MS (Duchefa) with 1% sucrose and 0.8% bactoagar. Seeds were stratified on plate for 2-3 days in darkness at 4ºC and then transferred to growth chambers set at 22ºC and with 16-h-light/8-h-dark cycle using cool-white fluorescent light conditions (100 nmol $m^{-2}s^{-1}$) provided by incandescent tubes (PHILIPS TLD-D 30W/33-640 and SYLVANIA GRO-LUX F30W/GRO). For low light experiments, low light condition was obtained by covering plates with several layers of whatman paper until light intensity with lux meter is becoming 15 µmol $m^{-2}s^{-1}$. Dark experiments were performed in the same chamber, with wrapping plates using several layers of aluminium foil.

Methods for transforming.

Bacterial transformation.

Transformation of competent DH5α *E. Coli* cells was carried out by heat-shock as described in (Sambrook *et al.*, 1989) or by electroporation as described in (Chassy and Flickinger, 1987). Competent cells were prepared through a calcium chloride treatment (Hanahan, 1985). Transformation of competent C58C1 *A. tumefaciens* cells was carried out as described in (Weigel and Glazebrook, 2002). Competent cells were generated through a freezing method

using also calcium chloride (Holsters *et al.*, 1978). Transformed *E. Coli* and *A. tumefaciens* cells were plated onto selective media (LB with corresponding antibiotics) and then, incubated overnight at 37 ºC or 48 hours at 28 ºC, respectively.

Transformation of Arabidopsis cell suspension cultures (PSB-D).

PSB-D Cell suspension cultures of Arabidopsis were grown on MSMO medium (4.43 g/L MS-minimal organics Sigma, 30 g/L glucose, 0.5 mg/L NAA, 0.05 mg/L kinetin, pH 5.7), in darkness (flasks were wrapped by aluminum paper) with constantly agitation at 130 rpm. The growth temperature was 25ºC. Generated lines were also maintained in solid MSMO medium (MSMO supplemented with 0.8% agar).

For transformation of the cultures, cells were co-cultured for 72 hours with A. tumefaciens C58C1 pMP90 containing corresponding construct, as is described in (Van Leene *et al.*, 2011). The selection of the transformed cells was carried out using MSMO supplemented with carbenicillin antibiotics (500 μg/mL) and vancomycin (500 mg/mL) for 3 weeks and decreasing concentrations of kanamycin: 50 μg/mL during the first 3 weeks and 25 μg/mL during the fourth week.

The cell suspension cultures were refreshed with fresh medium every 7 days. For TAP experiments material was collected 3 days after refreshing the medium.

Transformation of Arabidopsis thaliana.

For transformation of *Arabidopsis*, plants were grown in soil during 20-25 days in long-day conditions. Young inflorescences were infiltrated by inversion during 10 minutes with a suspension of *A. tumefaciens* carrying the construct of interest, in Murashige and Skoog (MS) medium (3.67 g of MS from Duchefa Bochemie per 1.5 L bidistilled water) supplemented with 5 % of sucrose and a 0.02 % of the surfactant agent Silwet L77 (Bechtold *et al*., 1993). Seeds obtained were sown in MS medium supplemented 50 μg/mL carbenicilin (used for excluding *A. tumefaciens* growing) and with the corresponding antibiotic for selection.

Agroinfiltration of *N. benthamiana* leaves.

N. benthamiana plants, provided by the CNB greenhouse service, grown during 3-4 weeks were infiltrated for BiFC experiments with the *Agrobacterium tumefaciens* C58C1 cells transformed with different constructs. Plasmid constructs were transformed by heat shock

into this strain according to (Endo *et al*., 2005), grown overnight and used for infiltration of the abaxial side of *N*. *benthamiana* leaves according to (Liu *et al*., 2010). Leaves were co-infiltrated with p19, which suppresses gene silencing (Voinnet, 2003). After 3 days of infiltration, leaves were used for confocal microscopic observations.

BiFC (Bi-molecular Fluorescent Complementation).

To carry out the BiFC experiments, proteins in study were inserted by LR-reaction (Invitrogen) from the entry vector (table 1) into the binary pBiFC vectors containing the N- and C- terminal YFP fragments). BiFC constructs used in this work are listed in table 5. To test the interaction, construcs were transformed into *A*. *tumefaciens* (C58C1) cells and co-infiltrated with p19, which suppress gene silencing (Voinnet, 2003), in *N*. *benthamiana* leaves. Leaves were observed by confocal imaging after 3 days. Positive nuclei were photographed. Leaf discs were immersed for a few minutes in a solution with DAPI to visualize nuclei (1 mg / mL 4,6-diamidino-2-phenylindole (DAPI) in 100 mM phosphate buffer and 0.5% triton 100).

Cross-fertilisation of *Arabidopsis thaliana*.

To obtain seeds and for cross-fertilisation, seedlings were transplanted into a mix of soil and vermiculite (ratio 3:1). Growing was carried out in the greenhouse at 22 ºC under long day conditions (16 h light/8 h dark) or short-day conditions (8 h light/16 h dark). *Arabidopsis* plants were grown in long-day conditions until the flowers were at a stage where they were not open, yet not closed. With some forceps, all flower parts except the ovary were removed, and with pollen obtained from a donor plant this ovary was pollinated. Once siliques were dry, seeds were harvested and grown for selection.

Hypocotyl length phenotype and measurements.

To determine seedling's hypocotyl length, seeds were plated in solid MS medium and grown horizontal or vertically. For horizontal plates 0.6% agar and 1% of sucrose was used, whereas vertical plates were prepared with 1% agar and without sucrose, in order to increase the texture strength of the media and avoiding root expanding. For darkness treatments, seeds were left for 6 hours under light conditions after stratification, and then plates were wrapped by various layers of aluminium foil. Seedlings were photographed at different days after sowing (growth kinetics) or at day 3 of growth for measuring the length of the hypocotyls from the apical meristem to the point of transition to the root. The average and standard deviations

of at least 10 plants were plotted for each experiment. Images were analysed with the *ImageJ* processing program (rsbweb.nih.gov/ij/).

Nucleic acid analysis and extraction.

Plant DNA isolation.

A. thaliana genomic DNA extraction was carried out following the method defined by (Doyle and Doyle, 1990).

Genotyping of Arabidopsis mutant lines.

To genotype lines carrying T-DNA insertions (*sgf11-1*, *sgf11-2, ubp22*) or point mutations (*det1-1* and *hy5-215)* (Table 1) we amplified DNA fragments containing the mutation by PCR using the thermostable polymerase Taq (Roche) and the primers listed in table 8.

In the *sgf11-1* mutants, the T-DNA is inserted in the promoter, which will generate a line without expression for this transcript. Amplification was performed using primers: 19 + 20 to amplify 800bp PCR fragment of the endogenous gene and 19+ SAIL (21) to amplify 500 bp PCR fragment of the T-DNA (table 8). Horizontal agarose gels (1%) were used with "SYBR safe DNA gel stain" (Invitrogen) in TAE buffer (50 mM Tris-acetate, 1 mM EDTA), to analyze the result.

In the case of ubp22 mutant, the T-DNA is inserted in 3rd exon, which will generate a line without expression for this transcript. For genotyping, amplification was performed using primers: 22 + 23 to amplify 850 bp PCR fragment of the endogenous gene and 22+ GK-08409 (24) to amplify 350 bp PCR fragment of the T-DNA (table 8). Horizontal agarose gels with "SYBR safe DNA gel stain" were used to analyze the result.

det1-1 mutant has a point mutation in the first intron affecting the processing site, creating a truncated protein. To genotype lines carrying the *det1-1* mutation, a 214 bp PCR fragment that includes this mutation was amplified by using primers *25* and 26 (table 8). The PCR products were then sequenced comparing to *det-1* and wild type plants. Plants including DNA sequence TCATTCC were selected comparing to DNA sequence have TCACTCC in wild type plants.

In *hy5-215* mutant the splicing acceptor site of the first intron (G) is replaced by A, suggesting that this mutation causes aberrant RNA processing. To select plant carrying *hy5* mutant, the 283 bp PCR products obtained with primers 27 and 28 were sequenced compared to *hy5* and

wild type plants. plants including DNA sequence AAAAGA were selected comparing to DNA sequence have AAAGGA in wild type plants (Table 8).

Table 8. Primers used for genotyping Arabidopsis mutant plants in this study.

No.	Insertion	Primer sequence
10	SGF11-SAIL 856 F11 F	ATGTCTGGCGCAGAGGATAATAAAT
11	SGF11-SAIL 856 F11 F	TCAGTCTCCTTTCACGTTCTCTCG
12	LB3-SAIL	TAGCATCTGAATTTCATAACCAATCTCGTACAC
13	GB_263H06 UBP22 LP	TTAACACGCTAACCTGATCCG
14	GB_263H06 UBP22 RP	GCGAAATGAAGAACTCGTGAG
15	GK_08409	ATATTGACCATCATACTCATTGC
16	*det1-1 F*	AACCTTCCTTTATTCGTGCC
17	*det1-1 R*	CGTAGAAGTGTCTTGCAC
18	*hy5-215 F*	CTGCTCCACATTTGGAGATCAAAG
19	*hy5-215 R*	CTCGCCGTATCTCCTCATCGCTTTC

Anthocyanins quantitation.

The Arabidopsis seedling extraction and anthocyanins quantitation was performed as described by (Ronchi *et al*., 1997) with minor modifications: 10 seedlings 3 days-old were incubated for 24 hours at 4ºC in 1 mL of HCl 0,5 N (v/v) in 25% methanol. Then seedlings were transferred to a cuvette (containing 0.5 mL of HCl 3N (v/v) in 16.5% methanol), vortex, and incubated for 15 min at room temperature. The absorption was determined spectrophotometrically A530 and A657 nm. As blank we used 0.5 ml of H_2O_2 30% was added to 0.5 ml of HCl 3N (v/v) in 16.5% methanol.

Mean values were obtained from three independent replicates. The anthocyanin content was calculated according to the next equation.

Anthocyanin content (mg/mg fresh weight) = A530 - 0.25(A657) X 2 (dilution factor)/mg fresh weight.

RNA preparation and quantitative real-time PCR analysis.

To analyze the expression level of *SGF11, FLC, FT, CO, MAF-4, MAF-5, HY5* and *MYC-SGF11,* total RNA was extracted (in triplicate) from around 20 6 day-old seedlings (or 3 day-old in case

of *HY5* transcripts) using the kit "RNeasy plant mini kit" (Qiagen) following the manufacturer recommendations. Subsequently the digestion of DNA present in the samples were carried out with the "Ambion® TURBO DNA-free ™" (Life Technologies) kit. The obtained RNA was quantified by the NanoDrop. For cDNA synthesis, 2 µg of total RNA was used for transcription with the "High-Capacity cDNA Reverse Transcription kit" (Applied Biosystems). The cDNA reaction was diluted 1:10 in water and used for qPCR analysis with primers specific to the desired genes (table 9). The "FastStart Universal SYBR Green Master mix" (ROCHE) was used for amplification, and primers specific for Actin-8 gene were used as internal control (table 9). The qRT-PCR reactions were performed in 96 well optical plates in a thermocycler "7500 Real-Time PCR system" (Applied Biosystems) and the amplification data analyzed by the 7500 software v2.0. PCR conditions were: 2 min at 50° C, 10 min at 95° C, and 40 cycles of 15 seconds at 95° C and 30 seconds at 60° C.

Table 9. Primers used to analyze gene expression in this study.

No.	Insertion	Primer sequence
20	F-qRT-SGF11	TGCCAAGACAACCAGAAGCA
21	R-qRT-SGF11	TCTTCACCTGCAACACCAGG
22	F-qRT-FLC	TTCTCCAAACGTCGCAACGGTCTC
23	R-qRT-FLC	GATTTGTCCAGCAGGTGACATCTC
24	MAF4-qRT- F	TGGCCAAGATCCTCAGTCGTTATGA
25	MAF4-qRT- R	GCTGCTCTTCCAGGGACTTTAGACA
26	MAF5-qRT- F	GATGGAGCTTGTGAAGAACCTTCAGG
27	MAF5-qRT- R	CAGCCGTTGATGATTGGTGGTTACTTG
28	HY5-qRT-F	CATCAAGCAGCGAGAGGTCA
29	HY5-qRT-R	CCGACAGCTTCTCCTCCAAA
30	MYC-SGF11-qRT-F	GTAGCGCTGTTATCACAAGTT
31	MYC-SGF11-qRT-R	CACCGAATCCACAAGATCAAG
32	ACTINA8-RT F	GGTACTGGAATGGTTAAGGC
33	ACTINA8-RT R	GTCCAACACAATACCGGTTG

Confocal Microscopy analysis.

For BiFC experiments in N. benthamiana leaves and for subcellular localization studies of GFP-SGF11, GFP-ENY2 and GFP-UBP22 lines (Table 1), we used Leica TCS SP5 mycroscope (Leica Microsystems) with a 63x water-immersion objective or 20x-dry objective. LAS AF v.2.3.6

software was used for image acquisition. Image processing was done with ImageJ software. We visualised root epidermal cells in *A. thaliana* from 4 days-old seedlings growing on vertical plates and epidermal cells of the underside of leaves in *N. benthamiana* plants.

Protein extraction from Arabidopsis seedlings and Western blotting analysis.

Seedlings were frozen and then homogenised in extraction buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM PMSF, 0.1 % NP-40 and and 1x complete protease inhibitor (Roche) using a stick "pellet pestle blue" (Sigma). Extracts were always kept on ice and clarified by centrifugation at 13000 rpm during 10 min at 4ºC. Supernatants were carefully collected into a new tube and centrifuged again for 10 min at 13000 rpm to remove all plant debris. This second supernatant was transferred to a new tube and the protein content was quantified by the Bradford protein assay method (Bio-Rad). Protein samples extracted were heated at 99 ° C for denaturation for 5 minutes in loading buffer 2x (63 mM Tris-HCl pH 6.8, 2% SDS (w/v), 20% Glycerol, 125 mM bromophenol blue, 3 mM 1,4 ditiotreitol and 10 % β-mercaptoethanol) and separated in 7 - 15% SDS-PAGE gels depends on the target protein size (Sambrook *et al*., 1989).

Gels were transferred to a PVDF membrane using a semidry transfer blot system. They were then saturated with 5% non-fat milk diluted in PBS buffer 0.1% Tween-20 (PBS-T) and used for immunodetection with the appropriate antibodies (Table 10) diluted in 1x milk in PBS-T buffer was incubated for one hour for peroxidase (or HRP) conjugated antibodies. In the case of the non-conjugated antibodies, the incubation was performed for 3 hours at room temperature or overnight at 4° C, followed by 3 washes of 10 minutes with PBS-T and incubation for one hour at room temperature with diluted secondary antibody 1: 10,000 in 1x PBS-T milk.

The secondary antibodies used were: Anti-Mouse IgG (Amersham Bioscience) and Anti-Rabbit (GE Healthcare). After this second incubation, the membranes were again washed as indicated above. As a detection system, "ECL Plus Western Blotting Detection System" (Amersham) or "SuperSignal® West Femto Maximum Sensitivity Chemiluminescent Substrate" (Thermo Scientific) was used according to the manufacturer's instructions. Most of the membranes where cut below 42 kDa for incubation with the RPT5 antibody or red Ponceau, which were used as loading control.

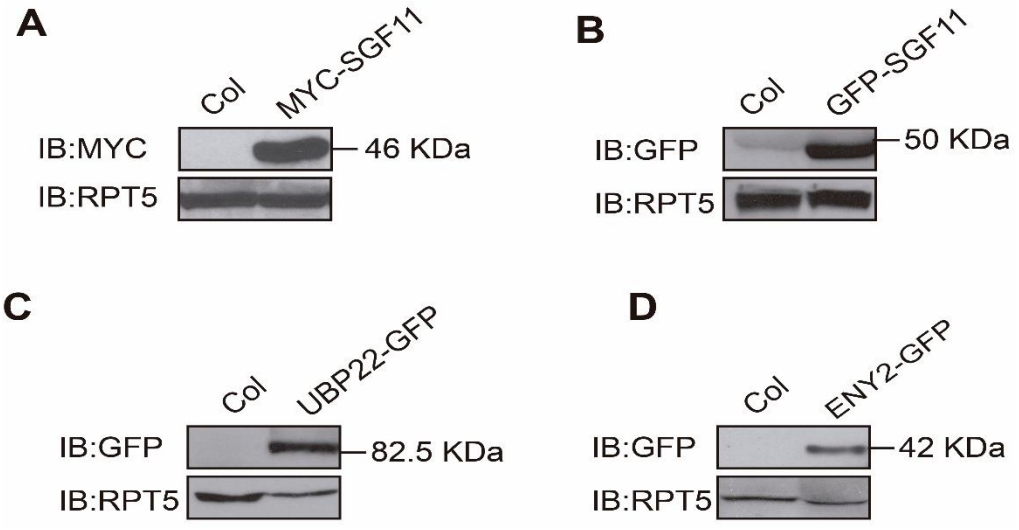


Figure 6. Protein expression level of generated overexpression lines. A) Protein level of 35S::MYC-SGF11. B) Protein level of 35S::GFP-SGF11. C) Protein level of 35S::UBP22-GFP. C) Protein level of 35S::ENY2-GFP RPT5 prtein was used as loading control to normalize protein levels.

Table 10. Antibodies used for Western blotting experiments.

Antibody	Dilution	Detection	Secondry	describtion
anti-GFP-HRP	1:2000	DDA1-GFP y DET1-GFP		Milteny Biotec
anti- PAP	1:1000	TAP-SGF11 de *Arabidopsis*		Sigma-Aldrich
anti- CUL4	1:2000	CUL4 de *Arabidopsis*	Anti-Rabbit	Chen *et al*., 2006
Anti-ubiquitina	1:1000	Molécula de ubiquitina	Anti-Rabbit	Boston Biochem
Anti-SBP	1:2000		Anti-Rabbit	Sigma-Aldrich
Anti-HY5	1:300		Anti-Rabbit	
Anti-H2B	1:1000		Anti-Rabbit	
Anti-MYC	1:1000		Anti-Mouse	
Anti-H2Bub	1:1000		Anti-Mouse	
Anti-CSN5	1:1000	CSN5 de *Arabidopsis*	Anti-Rabbit	Kwok *et al*., 1998
Anti-RPT5	1:1000		Anti-Rabbit	Kwok *et al*., 1999

Co-immunoprecipitation Assays.

Co-immunoprecipitation assays were carried out using 6 day-old Arabidopsis seedlings expressing GFP-SGF11, DET1-GFP, DDA1-GFP and MYC-SGF11. Col plants were used as negative control. Seedlings were carefully collected with forceps and incubated overnight in

liquid MS medium supplemented with 50 µM MG132 (Sigma-Aldrich). After treatment, the excess liquid was removed with blotting paper, and plants were frozen in liquid nitrogen and stored at -80 until use.

The concentration and volume extracted from approximately 80 seedlings with CoIP extraction buffer (50 mM tris pH 7.4, 80 mM NaCl, 10% glycerol, 0.2% NP40, 1 mM PMSF, 50 µM MG132, 1 µM DTT, 5 mM ATP and 1x protease inhibitors (Roche)) was normalized with the corresponding extraction buffer. To immunoprecipitate DDA1-GFP and DET1-GFP, protein extracts were incubated with 5 µL of anti-GFP (Living Colors Full-Length A: V: polyclonal antibody, Clontech) for 2 hours at 4 ° C with rotation, followed by incubation for 2 hours with 20 µl resin "Protein A-Sepharose ® from Staphylococcus aureus" (Sigma-Aldrich). Before use, the "Protein A-Sepharose" resin was treated with 0.1 M Glycine, pH 2.7, and washed 3 times with 1 mL of extraction buffer. To immunoprecipitate MYC-SGF11, protein extracts were incubated with “EZview™ Red Anti-c-Myc Affinity Gel” (Sigma) for 2-3 hours. To separate the resin unbound protein extract, wewashed three times with 1 mL of extraction buffer, and resin bound proteins were eluted by boiling 5 minutes in loading buffer 2x (63 mM Tris-HCl pH 6.8, 2% SDS (w/v), 20% Glycerol, 125 mM bromophenol blue, 3 mM 1,4-ditiotreitol and 10 % β-mercaptoethanol).

Samples were separated in 7 - 15% SDS-PAGE gels, transferred to a PVDF membrane and incubated with the appropriate antibodies (Table 10), as described before.

Protein extraction from Arabidopsis Cell suspension cultures (PSB-D).

For checking the expression of cell suspensions cultured transformed with Gsrhino-SGF11, between 3 and 15 grams of cell suspensions of Arabidopsis were homogenized using a mortar and liquid nitrogen. The milled material was mixed with the extraction buffer (containing 25 mM Tris-HCl pH 7.6, 15 mM MgCl2, 150 mM NaCl, 15 mM p-nitrophenyl phosphate, 60 mM β-glycerophosphate, 0.1% NP-40, 0.1 mM Na3VO4, 1 mM NaF, 1 mM PMSF, 1 µM E64, 1/10 mL of cocktail "Complete EDTA-free Ultra Tablet", 5% ethylene glycol and 2500 units of benzonase) using a coffee grinder precooled, and incubated for half an hour at 4 ° C with rotation. Extracts were centrifuged twice at 16,000 g for 10 minutes at 4 ° C to prevent protein degradation, and protein concentration in the supernatant was determined by Bradford (Bio-Rad Protein Assay Kit) by comparison with a standard curve (Van Leene *et al.*, 2015).

Tandem affinity purification (TAP)

TAP experiment was done from 2 liters of cell suspensions overexpressing Gsrhino-SGF11 fusion (i.e. two repeats of the G protein, a cleavage site for 3C protease rhinovirus and binding site SBP, all under control a constitutive promoter 35S; see section “Generation of expression vectors in Arabidopsis cell cultures line (PSB-D)”. TAP experiment consist in protein extraction (explained before), followed by a first step of purification with immunoglobulin G affinity resin "IgG Sepharose FF" (GE healthcare), incubation with protease "Rhinovirus 3C protease" (GE healthcare) that cuts at 4 ° C, and a second purification step with the streptavidin binding resin "Streptavidin Sepharose" (GE healthcare), that required desthiobiotin for elution, as described by (Van Leene *et al*., 2015).

For the identification of proteins present in the final eluate, proteins were separated on a NuPAGE gradient gel 4-12% NuPAGE Bis-Tris (Invitrogen), followed by trypsin digestion as described by (Van Leene *et al*., 2011). Peptides were analyzed with a nano-LC system connected to a mass spectrometer LTQ Orbitrap Velos (Thermo-Fisher), and identificatified by TAIRplus it database. These analyses were conducted in collaboration with Dr. Geert de Jaeger laboratory (VIB, Ghent, BELGIUM). The contaminants or background proteins were discarded based on comparison with databases of protein present in other assays available in the laboratory of Dr. Geert Jaeger.

Histone extraction.

Histone extraction from plants was carried out in two steps, nuclei extraction followed by another step to extract histone extraction from nucleus.

Nucleus extraction.

To extract histones from Arabidopsis seedlings 6-day-old seedlings, approximately 3 g of material were ground to a fine powder and homogenized for 15 min in 30 ml of nucleus extraction buffer1 (0.4 M Sucrose, 10 mM Tris–HCl, pH 8.0,10 mM MgCl2, 5 mMβ-ME and 0.1 mM PMSF). After filtertion through two layers of miracloth in 50 ml falcon tube. The flitered was centrifugation for 20 min at 4°C and 4000rpm, pellets were dissolved in 1 ml of nucleus extraction buffer2 (0.25 M Sucrose, 10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1% Triton X-100, 5 mMβ-ME and 0.1 mM PMSF). After centrifugation for 10 min at 4°C and 11000 rpm at 4°C for 10 min, the nucleus pellet obtained was whitish-gray.

Histones extraction from nucleus.

pellets were dissolved in 0.1 MH_2SO_4. After further centrifugation for 10 min at 17,000*g*, total histones were precipitated from the supernatant with concentrated trichloroacetic acid to a final concentration of 33%. The histone pellet was washed twice with acetone-0.1% HCl, vacuum-dry, dissolved in H_2O. the Histone was quantified by Brad-Ford at 595 nm. 10 µg was subjected to SDS-PAGE, followed by protein immunoblotting using anti-H2Bub (07-690; Upstate/Millipore) and anti-H2B (39379; ActiveMotif) antibodies.

Protein extraction buffer: 50 mM tris-HCl, 150 mM NaCl, 10 mM MgCl2, 1 mM PMSF, 0.1% NP40 and 1x inhibitors protease (Roche).

loading buffer 2x: 63 mM Tris-HCl pH 6.8, 2% SDS (w / v), 20% glycerol, 125 mM bromophenol blue and 3 mM dithiothreitol and 1,4-10% β-mercaptoethanol.

Affinity purification of ubiquitinated proteins.

For purification of ubiquitinated proteins, seedlings MYC-SGF11, det1-1/oeMYC-SGF11 and Col 6 day-old were collected with forceps from MS plates, and transferred incubated overnight in MS liquid medium containing 50 µM MG132. After treatment the plants were dried with paper tissue, frozen in liquid nitrogen and stored at -80 ° C until use.

The process of extraction and purification of proteins was performed as described by (Manzano *et al.*, 2008) with some modifications. Homogenized seedlings were mixed with extraction buffer BI (50 mM Tris-HCl pH 7.5, 20 mM NaCl, 0.1% NP40, 5 mM ATP, 1x protease inhibitors (Roche), 1 mM PMSF, 50 µM MG132, 10 nM ub aldehyde (Enzo Life Science) and 10 mM N-ethylamide). The extracts were centrifuged twice for 10 min at 4 ° C, and the supernatants were normalized concentration and volume with their respective controls, after assessing concentration by Bradford using standard curve.

After removing a small volume (40 µg) of each extract (kept as loading control, INPUT), each sample was incubated for four hours at 4° C in rotation with 40 µl resin "p62 agarose" (Wilkinson *et al.*, 2001) previously washed with extraction buffer. For the negative controls only agarose resin was added. After the incubation we proceeded to wash the resin twice with 1 mL of BI buffer, followed by 1 wash with 1 mL of BII buffer (same composition as the BI buffer 200 mM NaCl). Bound proteins were eluted from resin by heating for 5 minutes at 99 ° C

in 50 µl of 2x loading buffer.

The extracts were separated on two parallel SDS-PAGE gels. Among of 50 µl, 10 µl were loaded in one gel and 40 µl in the another gel. Both were transferred to PVDF membrane. 10 µl gel was immunoblotted with anti-ubiquitin antibody (to detect the presence of ubiquitinated proteins), whereas 40 µl gel was incubated by anti-MYC for detection of protein MYC-SGF11.

In vivo degradation assay.

6 day-old seedlings growing under light and dark conditions of oeMYC-SGF11, det1-1/oeMYC-SGF11 and Col, were transferred to liquid MS medium plate containing 50 µM CLX under conditions for 1, 2, 3 and 4 hours. Plants were also collected untreated as control initial time (T0). The effect of proteasome inhibition was checked by adding 50 µM MG132 after 4 hours of incubation. Protein extraction of 40 treated plants was carried out as indicated previously in "Protein extraction from Arabidopsis seedlings and Western blotting analysis". Protein extracts were separated on SDS-PAGE gels and transferred to PVDF membrane, then immunoblotted by anti-MYC and as loading control membranes with red stain Ponceau and anti-RPT5. ImageJ version 1.37 software was used to analyze the intensity of the bands.

In vivo degradation of plants under Light shift to dark /dark shift to light conditions.

To check if the degradation level correlates with the results obtained with plants grow under light conditions, 6 day-old seedlings growing under light and dark conditions of oeMYC-SGF11, det1-1/oeMYC-SGF11 and Col were shifted to dark or light respectively. Seedlings were collected for protein extraction comparing to initial time (T0), which is total light or total dark respectively. Proteins were separated in SDS-PAGE gels and transferred to PVDF membrane, then immunoblotted by anti-MYC and as loading control membranes with red stain Ponceau and anti-Rpt5, as described before.

Functional complementation assays of overexpression lines.

To determine the functionality of SGF11 in oeMYC-SGF11 overexpression lines, we check the mono-ubiquitination H2B level by western blot in lines. *sgf11-1*/oeMYC-SGF11, *sgf11-1*/oeGFP-SGF11. 6 day-old seedlings grown under long day conditions were collected and histones extraction was done. 10 µg of the total histones were loaded into 15% SDS-PAGE gel, the

proteins were transferred to PVDF membrane and immunoblotted with anti-H2Bub antibody to detect a 27KDa band. The lower part of the membrane was immunoblotted with anti-H2B to detect a band around 17 KDa as loading control.

RESULTS

CHAPTER I: Search for DDA1 interactors identifies a novel protein involved in chromatin remodelling in *Arabidopsis.*

Introduction.

In order to elucidate the molecular mechanisms by which DDA1 contributes to the activity of CRL4-CDDD E3 ligases, a search for DDA1 protein interactors was previously carried out (Irigoyen *et al*., 2014). Several putative DDA1-interacting proteins were identified in a yeast two-hybrid screen using an *Arabidopsis* cDNA library and DDA1 as a bait. Functional characterization of two of these interactors shed light on the biochemical function of DDA1 protein. Thus, it was found that DDA1 negatively regulates abscisic acid (ABA)-mediated developmental responses by recognizing and targeting for ubiquitination and subsequent degradation members of PYR/PYL family of ABA receptors. In this way, DDA1, as part of the CDDD module, acts as a novel type of substrate adaptor for CRL4 E3 ubiquitin ligases (Irigoyen *et al*., 2014). In the present study, we aim to evaluate the physiological relevance of an additional DDA1-protein interaction identified in the same yeast two-hybrid screen, that of DDA1 with SAGA-ASSOCIATED FACTOR 11 (SGF11). The later has been described as an 11-kDa subunit of the SAGA nuclear protein complex in both yeast and animal systems (termed ATNX3L7). SGF11 together with ubiquitin protease Ubp8 are constituents of the deubiquitination module (DUBm) of the SAGA complex, which removes ubiquitin marks from H2B in *Saccharomyces cerevisiae* (Ingvarsdottir *et al*., 2005). H2B ubiquitination has been shown to facilitate high and fast responsiveness to light of a significant number of genes that are negatively regulated by CRL4-CDDD complexes under dark conditions (Bourbousse *et al*., 2012). The fact that DDA1 interacts with SGF11 provides a plausible scenario in which CRL4-CDDD complexes may control the activity of the DUBm in order to control H2B ubiquitination, and therefore light-responsive gene expression. The main objective of the present study is to test the validity of this idea.

DDA1 interacts with SAGA-ASSOCIATED FACTOR 11 (SGF11).

In order to shed light on the mechanisms of action of DDA1, a search for proteins that physically interact with DDA1 was performed previously in our laboratory using the yeast two-hybrid system (Irigoyen *et al*., 2014). For this, a construct containing the full length cDNA of *DDA1* fused to the binding domain of *GAL4* (pGBKT7-DDA1) was generated and cloned into the Y187 strain. The resulting yeast clone was used as bait to screen a library of Arabidopsis

seedlings cDNAs fused to the to the activator domain of *GAL4* (cloned into the pGADT7 vector and transformed into the AH109 strain). Upon yeast mating and selection of clones growing in histidine-free medium, 200 potential interactors of DDA1 were identified. Among them, 20 candidates were confirmed by yeast co-transformation experiments. Several candidates corresponded to proteins previously involved in different biological processes in plants, including:

-Cell cycle progression.
-Chromatin Remodelling.
-Response to various stresses.
-Seed desiccation.

Among these putative interactors, two clones were found corresponding to a truncated version of SAGA-ASSOCIATED FACTOR 11 (SGF11); spanning from aa to aa. Thus, as shown in Figure 7A, DDA1 strongly interacted with the truncated version of SGF11 in yeast cells, since cells co-transformed with these two constructs grew in selective media in the absence of all four amino acids leucine, tryptophan, histidine and adenine (SD-WLHA), whereas the corresponding negative controls, transformed with empty vectors, did not (Figure 7A).

Amino acid sequence comparison of the SGF11 orthologue from different organisms showed a conserved domain in the SGF11 protein, containing zinc finger motifs (ZnF) which are required for H2B deubiquitination by the DUBm (Koehler *et al*., 2014)., This ZnF motif was missing in the truncated versions of SGF11 isolated in our yeast two hybrid screens (figure 7 A). To test whether DDA1 is able to interact with the full-length version of SGF11, the complete cDNA of *SGF11* was amplified by PCR out of wild type Col-0 plants and cloned into both pGBKT7 and pGADT7 vectors, to be used as bait and prey, respectively, in yeast tow hybrid assays. Already available DDA1 constructs in both pGBKT7 and pGADT7 vectors (Irigoyen *et al*., 2014) were used in these assays.

As seen in (Figure 7B), DDA1-SGF11 interaction was confirmed by growth of yeast clones in SD–WLHA medium in the presence of 3-Amino-1,2,4-triazole (3-AT), a competitive inhibitor of histidine biosynthesis. Interaction between these proteins suggested a novel link between CRL4-CDDD complexes and histone modification by DUBm that we aimed to characterize in vivo.

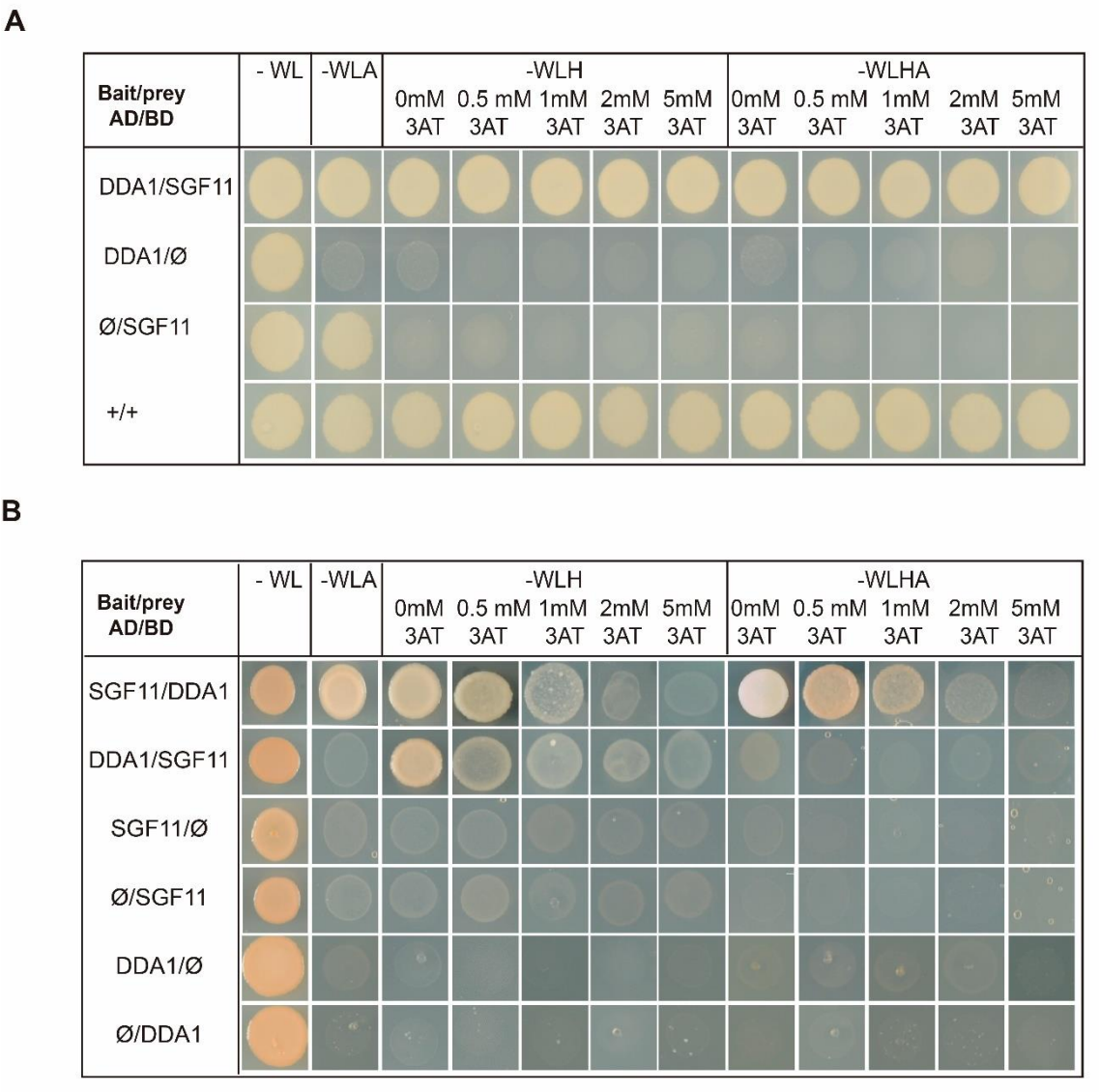


Figure 7. Yeast two-hybrid assays to test the interaction of DDA1 and SGF11 proteins. A) DDA1 interacts with a truncated version of SGF11, lacking the ZnF motif, in yeast cells. B) Protein-protein interaction of full-length versions of DDA1 and SGF11 in yeast cells. AH109 yeast cells co-transformed with bait and prey constructs, or with the corresponding empty vectors used as negative controls, were grown on SD-WL media as a control for co-transformation, and on SD-WLHA selection media to assay for protein interaction.

DDA1 and SGF11 proteins physically interact in vivo.

The fact that DDA1 binds to SGF11 points to a role of CRL4-CDDD in the control of SGF11 protein accumulation. Thus, DDA1 may recognize SGF11 as a substrate for ubiquitination mediated by CRL4-CDDD complexes and subsequent protasomal degradation, as previously described for PYR/PYL ABA receptors (Pick *et al*., 2007; Irigoyen *et al*., 2014). Such a mechanism may reveal a novel role of CRL4-CDDD complexes in the modulation of histone post-translational modification (H2B ubiquitination/deubiquitination) in specific genomic regions controlling different biological processes. To substantiate this regulatory model,

interaction of DDA1 and SGF11 proteins was tested first *in planta* by BiFC (Bimolecular Fluorescence Complementation assay). For this, SGF11 and DDA1 were each tagged with different YFP fragments (cYFP or nYFP) and co-expressed by agroinfiltration in *Nicotiana benthamiana* leaves. Nuclear fluorescent signal was detected in leaves co-expressing both protein fusions but not in negative controls expressing one of these fusions and the corresponding empty vector (Figure 8A and B). Thus, we can conclude that the SGF11 and DDA1 proteins interact in planta.

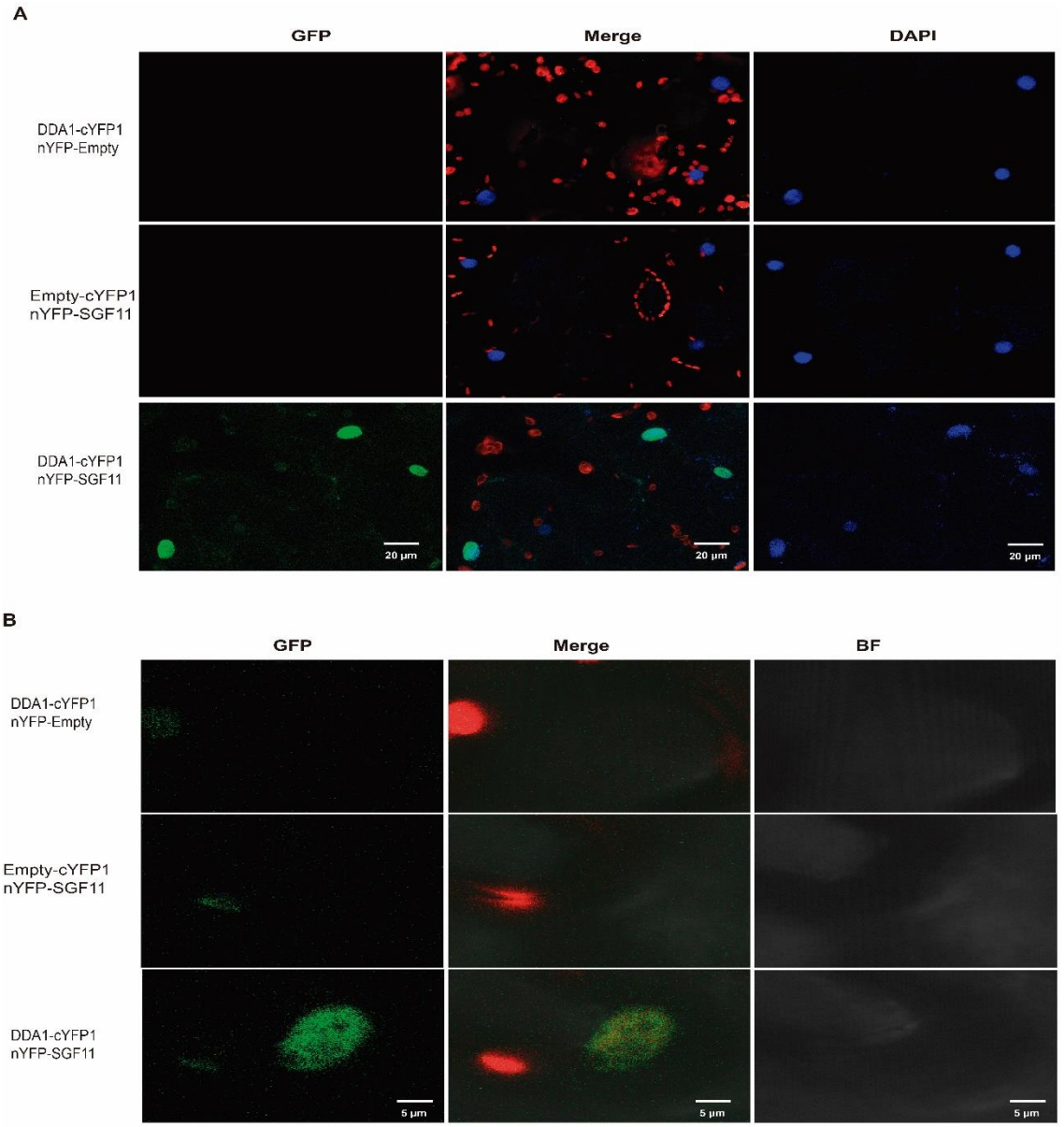


Figure 8. BiFC analyses of SGF11/DDA1 interaction in *N. benthamiana* leaves. A and B) Nuclear YFP fluorescence was detected in leaves infiltrated with nYFC-SGF11 and cYFC-DDA1 constructs, indicating physical interaction between both proteins. The interaction occurred in nuclei as confirmed by DAPI staining in Figure A. Agroinfiltration of each construct and the corresponding empty vector pair was used as a negative control. Bar=20 µm in A and Bar=20 µm in B. All images are shown at the same magnification.

SGF11 associates to CRL4-CDDD complexes.

To check whether SGF11 is able to interact directly with additional CDDD components, we carried out in vivo BiFC and yeast two-hybrid assays (Figure 9). For these, a series of constructs for all CDDD components, already available in our laboratory (Irigoyen *et al*., 2014), were used. As a result, no direct interaction was observed between SGF11 and DET1, DDB1A or DDB1B in yeast two-hybrid experiments. However, strong SGF11-COP10 interaction was observed while using the yeast two-hybrid system. To check whether the SGF11 and COP10 interact in vivo, BiFC analyses were carried out where both proteins were fused to cYFP and nYPF fragments and co-expressed in *N. benthamiana* leaves. As a positive control, the DDA1-SGF11 pair was used. Upon several attempts YFP fluorescence could not be visualized in leaves agroinfiltrated with SGF11 and COP10 fusions (Figure 9B). Thus, we conclude that SGF11 and COP10 proteins very likely do not interact in planta, and that SGF11 association mainly occurs through its interaction with DDA1. However, we cannot exclude the possibility that COP10 and SGF11 BiFC constructs were missexpressed in agroinflitrated *N. benthamiana* leaves.

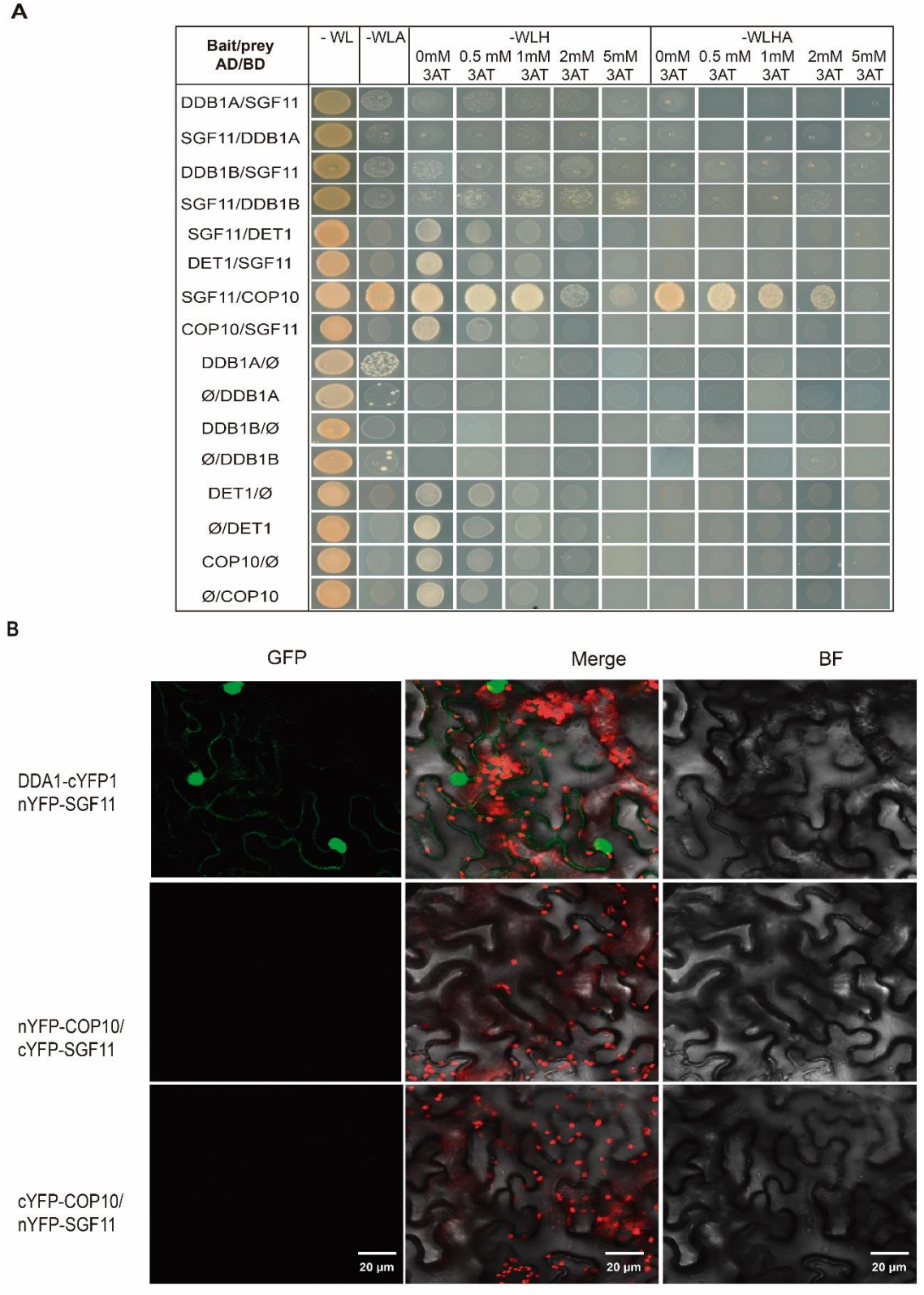


Figure 9. Among CDDD components, SGF11 solely interacts with DDA1 in vivo. A) SGF11- COP10 interaction was detected in yeast two hybrid assays but it could not be confirmed in vivo by using BiFC assays as shown in B). Nuclear YFP fluorescence from nYFC-SGF11/cYFC-DDA1 combination was used as a positive control. Negative controls corresponding to combinations with empty vectors are not shown for simplicity sake. Bar=20 µm. All images are shown at the same magnification.

It has been previously described by our group that DDA1 is part of the CDDD complex. The later acts as a substrate adaptor module for larger CRL4 complexes (CRL4-CDDD) (Irigoyen *et al*., 2014). Assembly of CRL4-CDDD complexes is regulated by the neddylation/deneddylation status of CUL4 (Iniesto *et al*., unpublished), a process that is controlled by the CSN complex. Therefore, CSN-mediated deneddylation of CUL4 triggers disassembly of CRL4-CDDD, which is essential for CDDD to interact with novel protein targets and bring them back to CRL4 for ubiquitination. To test whether SGF11 associates with CUL4 and CSN complexes in vivo, transgenic plants overexpressing N-terminal fusions of SGF11 to GFP (oeGFP-SGF11) and 10xMYC epitope (oeMYC-SGF11) were generated. First, we checked whether a GFP-SGF11 fusion is able to associate with CUL4 by performing pull-down assays using polyclonal anti-GFP and oeGFP-SGF11 protein extracts. As a result, we could detect CUL4 in GFP-SGF11 immunoprecipitates using specific anti-CUL4 antibodies (Chen *et al*., 2010;Figure 10A).

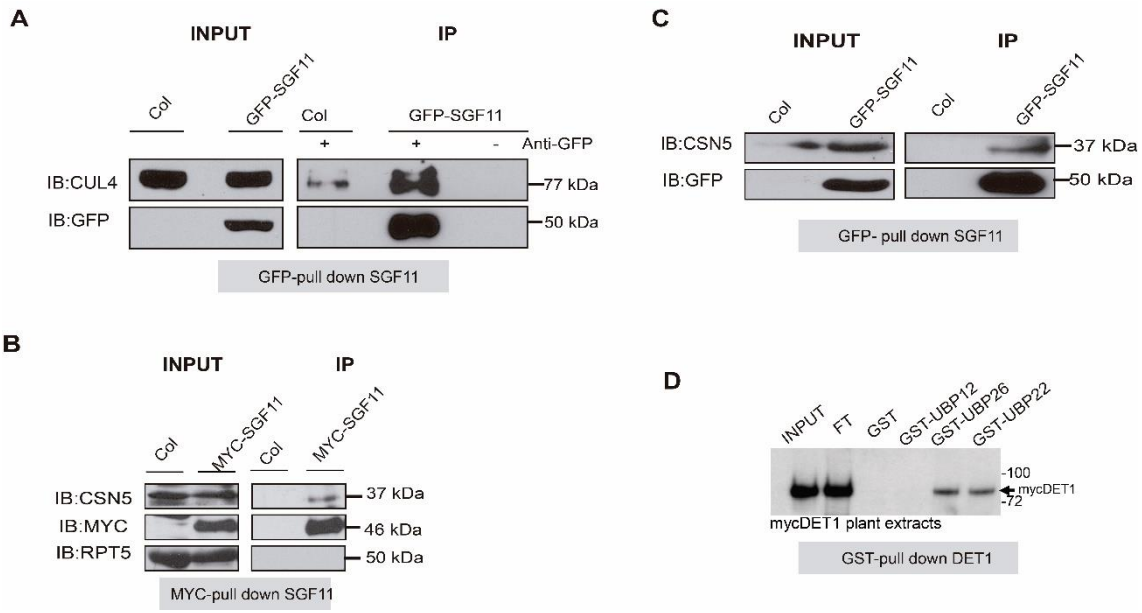


Figure 10. CRL4-CDDD and DUBm complex components associate in vivo. A) CUL4 protein coimmunoprecipates with GFP-SGF11 fusion. anti-GFP was used for immunoprecipitation and detection of the GFP-SGF11 fusion. CUL4 was detected by immunoblotting with anti-CUL4. Wild-type Columbia (Col) extract plus anti-GFP and oeGFP-SGF11 extracts incubated just with agarose resin were used as anegative controls. B and C) SGF11 was found to interact with CSN5, a key subunit of the CSN complex (Wang, 2009). A band corresponding to CSN5 was detected in both oeGFP-SGF11 and oeMYC-SGF11 immunoprecitates. anti-GFP and anti-Myc were used for immunoprecipation and fusion detection in B) and C), respectively (C) respectively. D) Semi in vivo precipitation assays using recombinant GST-DET1 assays and protein extracts from Arabidopsislines overexpressing myc-DDB1a indicate that DET1, in addition to DDB1 and CUL4 proteins, associates with H2B deubiquitinases UBP26 and UBP22. Anti-myc and specific antibodies against CUL4, HUB1/HUB2 and UBP26 were used for protein detection.

In addition, SGF11 was found to associate to CSN5 in vivo after immunoprecipitation of both

GFP-SGF11 and MYC-SGF11 fusions (Figure 10C and D respectively), using polyclonal anti-GFP and anti-c-Myc resins, respectively. In these assays, CSN5 was detected in immunoprecipitates by using commercially available anti-CSN5 antibody (Figure 10C and D). In order to further substantiate the link between CRL4-CDDD and DUBm complexes, additional protein-protein associations were tested using DET1 as a bait. For this, recombinant GST-DET1 was produced and purified from bacteria and incubated with Arabidopsis wilt-type protein extracts. Using Gluthation-sepharose resin, GST-DET1 proteins were pulled-down and precipitates were immunoblotted against different components of the H2B ubiquitination/deubiquitination machinery. As shown in Figure 10D, GST-DET1 associated with two different ubiquitin proteases; UBP22, where the later is also a component of DUBm complexes. However, no binding between GST-DET1 and H2B Ub ligases HUB1 and HUB2 was observed. This result suggests that CRL4-CDDD complexes may control H2B ubiquitination by specifically controlling the activity of H2B deubiquitinases.

Chapter II: Biochemical and functional characterization of the DeUBiquitination module (DUBm) in *Arabidopsis*.

Introduction.

The SAGA is a large protein complex composed of more than 20 subunits arranged in four modules that acts as a gene expression coactivator in different organisms. The SAGA complex has been highly conserved through the evolution of eukaryotes where it is involved in the regulation of numerous cellular processes through coordination of post-translational modification of various histones. Indeed, the SAGA complex has been shown to display both histone acetyltransferase and histone deubiquitination enzymatic activities in yeast and mammalian cells. (Helmlinger *et al*., 2004; Lee and Workman 2007; Weake and Workman 2012).

Gene candidates for most SAGA subunits have been identified in *Arabidopsis* and rice using an in silico approach, although functional information for most of them is still missing (Moraga and Aquea, 2015; Srivastava *et al*., 2015). The best-characterized subunit is the Arabidopsis homolog of GCN5, a member of the Gcn5-related N-acetyltransferases (GNAT) family, which mediates histone acetylation (Pandey *et al*., 2002; Chen and Tian, 2007). Similar to mammals and yeast counterparts, *Arabidopsis* GCN5 contains a bromodomain that is required for the binding of GCN5 to its target loci at the chromatin. Mutants in this gene display a global reduction in histone H3 acetylation (Stockinger *et al*., 2001; Mao *et al*., 2006; Earley *et al*., 2007). More specifically, histone H3K14 and H3K27 acetylation were reduced at defined loci in *Arabidopsis gcn5* mutants (Benhamed *et al*., 2006). Interestingly, analysis of GCN5-bound genomic fragments revealed significant overlap with HY5 target genes, suggesting a role for SAGA complexes in the control of photomorphogenesis (Lee *et al.*, 2007). However, little is known about the molecular mechanisms that allow SAGA-dependent deubiquitination of histone H2B in plants and its effect in plant biological processes.

Our study has been focused in the characterization of the so-called DeUBiquitination module (DUBm) in *Arabidopsis*, identifying its components and exploring their potential roles in plant development.

UBP22, SGF11, and ENY2 are components of the DUBm complex in *Arabidopsis*.

In silico identification of DUBm components in *Arabidopsis*.

In order to identify all DUBm components in *Arabidopsis*, an in silico approach was followed similar to that described in the studies by Moraga and Aquea, 2015, and Srivastava *et al*., 2015. Thus, TBLASTN searches (https://www.arabidopsis.org/Blast/index.jsp) were carried out using protein sequences for all DUBm subunits from *S. cerevisiae* and humans as queries against the *Arabidopsis* genome (TAIR). As a result, genes corresponding to At5g58575, At5g10790, and At3g27100 showed similarity to yeast and human Sgf11/ATXN7L3, Ubp8/USP22 and Sus1/ENY2, respectively. However, searches throughout the *Arabidopsis* genome did not yield any homologue genes for Sgf73/ATXN7. In yeast, Sgf73 acts as an anchor protein, connecting the DUBm to the rest of the SAGA complex (Lee *et al*., 2009). Our data suggest that the DUBm might function independently from SAGA in *Arabidopsis* or, alternatively, that other protein(s) could be involved in anchoring DUBm to SAGA in *Arabidopsis*.

The protein domains of candidates for DUBm subunits in *Arabidopsis* were aligned with the corresponding protein domains of S*. cerevisiae, D. melanogaster, H. sapiens* and *Oryza sativa* DUBm homologues, by direct ClustalW alignment and Box Shade amino acid comparison analysis (Figure 11). *Arabidopsis* SGF11, UBP22 and ENY2 shared high similarity at the conserved domains to proposed DUBm components in *S. cerevisiae, D. melanogaster and H. sapiens* (Figure 11). This indicates that the DUBm components are evolutionary conserved between *Arabidopsis*, yeast and mammals with the exception of SGF73.

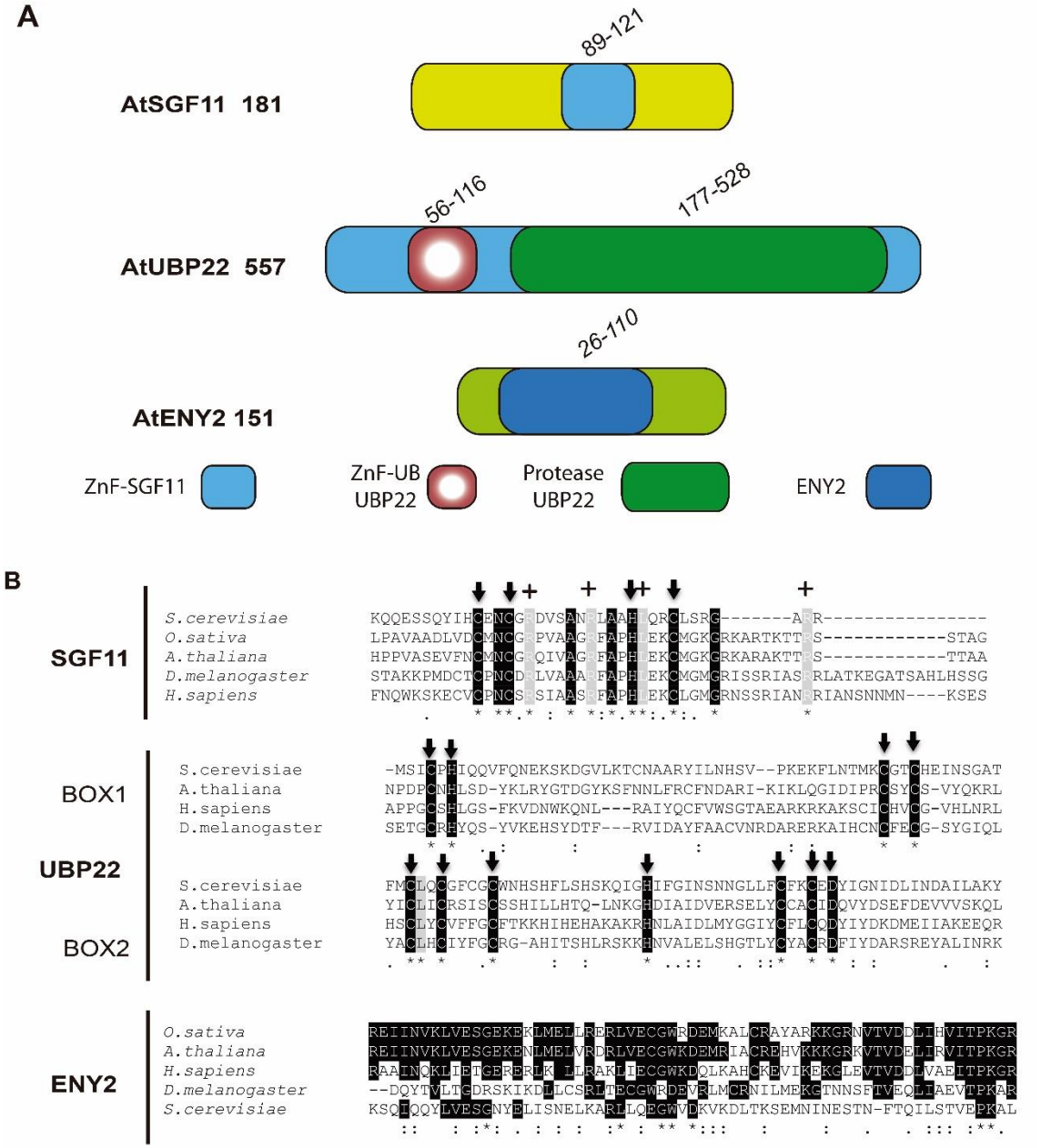


Figure 11. Domain organization of *Arabidopsis* DUBm homologs. A) Schematic representation of the expected DUBm in *Arabidopsis (*Srivastava *et al*., 2015). B) Position of conserved domains within Arabidopsis DUBm protein homologues. (C) Sequence alignment of the ZnF-Sgf11 and ZnF-UBP22 and Eny2-ENY2 domains from different organisms. Highly conserved residues (black) and positive charged DNA-binding residues (light gray) present in the corresponding homologues are highlighted. Arrows indicate core residues at Zn finger domains in SGF11- and UBP22-related proteins.

Arabidopsis SGF11 physically interacts with UBP22 and ENY2 homologs.

To determine whether SGF11 binds to other potential subunits of the *Arabidopsis* DUBm and to characterize their interaction network, we first performed yeast two-hybrid (Y2H) assays using all SGF11, UBP22 and ENY2 pair combinations. To this aim, the cDNAs of *AtSGF11*, *AtUBP22* and *AtENY2* genes were cloned into Y2H vectors. As shown in Figure 12,

both UBP22 and ENY2 showed strong interaction with SGF11. However, no interaction was observed between UBP22 and ENY2 proteins in yeast cells (Figure 12A and 12B), suggesting that SGF11 might be necessary for connecting UBP22 with ENY2.

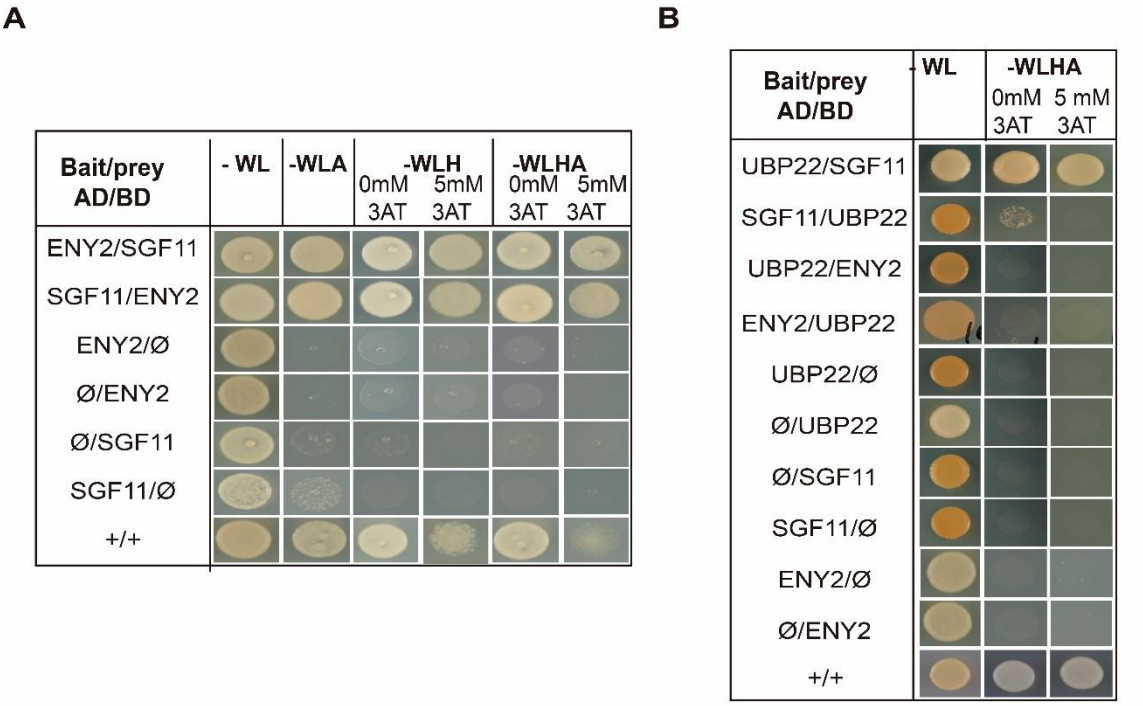


Figure 12. SGF11 binds directly to ENY2 and UBP22 proteins. A and B) SGF11 interacts with ENY2 and UBP22 proteins in yeast two-hybrid assays (Y2H). Yeast clones co-transformed with the indicated constructs were grown in selective medium (indicated at the top of the table) with different concentrations of 3AT (up to 5 mM).

Next, the interactions of UBP22, ENY2 and SGF11 with each other were tested in vivo using Bi-molecular complementation assays (BiFC; Figure 13). For this, fusions of these proteins to the nYFP and cYFP fragments of the YFP protein were co-expressed in epidermal cells of *N. benthamiana* leaves. Nuclear fluorescent signal was detected in cells co-expressing both SGF11 and UBP22 or SGF11 and ENY2 fusions but not in the negative controls expressing one of these fusions and the corresponding empty vector (Figure 13A and B). No signal was detected in cells co-expressing both SGF11 and UBP22 or ENY2(Figure 13B).

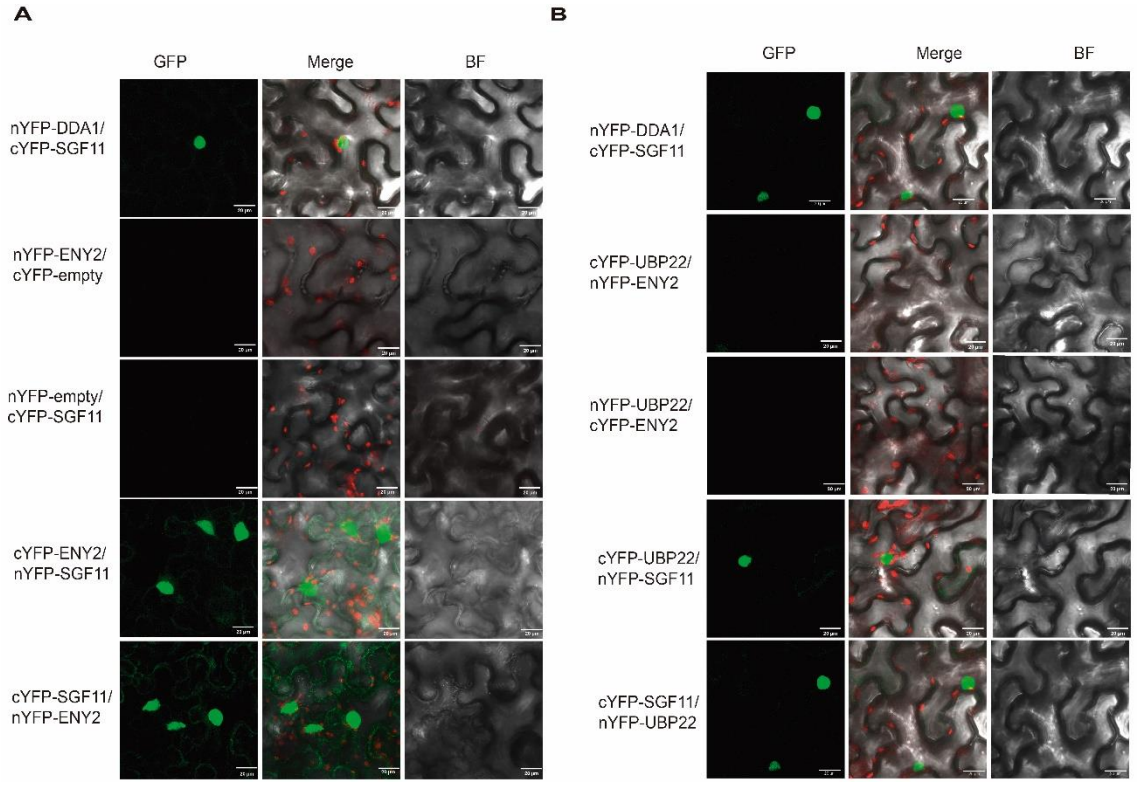


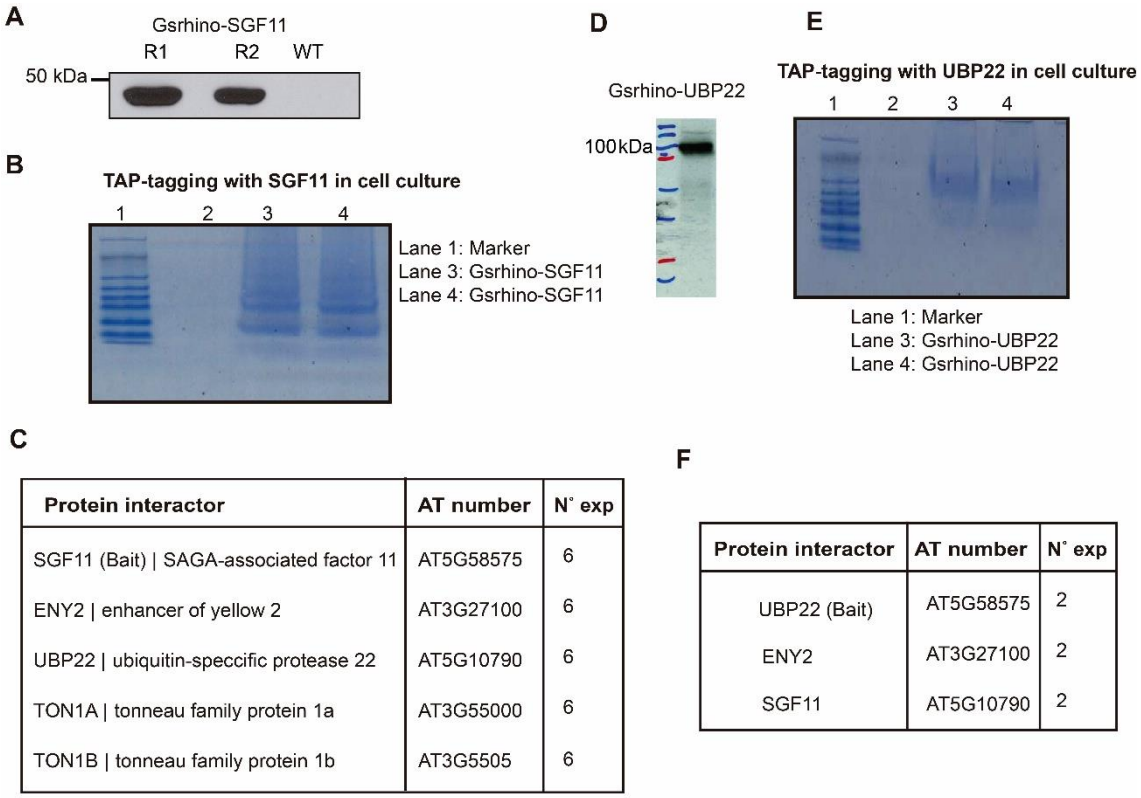
Figure 13. In vivo interaction between DUBm protein subunits. A and B) Nuclear YFP fluorescence was detected in leaves infiltrated with nYFP/cYFP–SGF11 and nYFP/cYFP–ENY2/UBP22 constructs and not with nYFP/cYFP– UBP22 and nYFP/cYFP–ENY2. YFP signal was not detected in leaves agroinfiltrated with empty vectors and each of the protein fusions (data not shown). Bar=20 µm. All images are shown at the same magnification.

SGF11, UBP22 and ENY2 are constituents of the DUBm complex in *Arabidopsis*.

Y2H assays and BiFC experiments indicate that SGF11 is able to physically interact with both UBP22 and ENY2. Next, we analyzed whether these three proteins associate as part of larger complexes such as the DUBm and the SAGA as it has been shown in yeast and mammalian systems. To this end, both SGF11 and UBP22 proteins were tagged to the GSrhino-tag which allows protein complex purification by using Tandem Affinity Purification techniques (TAP; REF) and expressed in Arabidopsis cell cultures (see Methods; Figure 14B). Upon TAP purification of both protein fusions, samples were subjected to mass spectrometry analysis and subsequent search of isolated peptides in the TAIR data plus database (http://www.psb.ugent.be/tap-fasta-datasets) for protein identification using MASCOT (http://www.matrixscience.com/) (experiments performed in collaboration with the group of Dr. Geert de Jaeger; VIB, Ghent, Belgium).

Purification of SGF11-associated proteins confirmed that SGF11 associates with UBP22 and ENY2 proteins in *Arabidopsis* cells as it does in yeast and mammalian cells to constitute the DUBm (Figure 14). According to our in silico data, no evidence was found for the existence of

an SGF73 homologue (Figure 14C), suggesting that either DUBm uses an additional protein(s) to interact with the rest of the SAGA complex or both complexes function independently in *Arabidopsis*.



C

Protein interactor	AT number	N° exp
SGF11 (Bait) | SAGA-associated factor 11	AT5G58575	6
ENY2 | enhancer of yellow 2	AT3G27100	6
UBP22 | ubiquitin-speccific protease 22	AT5G10790	6
TON1A | tonneau family protein 1a	AT3G55000	6
TON1B | tonneau family protein 1b	AT3G5505	6

F

Protein interactor	AT number	N° exp
UBP22 (Bait)	AT5G58575	2
ENY2	AT3G27100	2
SGF11	AT5G10790	2

Figure 14: Proteins identified as interactors of SGF11 and UBP22 in TAP assays. A and D) Immunoblotting analysis of GSrhino fuesd to SGF11 in (A) and UBP22 in (D) using anti-SRB antibodies. B and E) SDS-PAGE of the associated proteins after stained with Coomassie. B and E) TAP purified proteins were separated onto High-performance SDS-PAGE gels and stained with Coomassie-blue prior to in-gel trypsin digestion and MS identification of proteins associated with SGF11 (B) and UBP22 (E). C and F) Table indicating AGI (*Arabidopsis* Genome Initiative) codes and the names of co-purified proteins, along with the number of experiments in which they were found 6 times in SGF11 (C) and 2 times (F).

In this regard, mass spectrometry analyses identified additional proteins that co-purified with the SGF11 fusion and that have not been related to the DUBm in other organisms. This was the case of TONNEAU1A and 1B proteins. TON1A and TON1B contain LIS1 Homology (LisH) motifs and are involved in the microtubule and chromosomal rearrangements that precede cell division (Azimzadeh *et al*., 2008). However, TON1 proteins were not present in TAP samples where UBP22 was used as bait, suggesting that their association to the DUBm is transient or spurious. Whether this is the case or, on the contrary, TON1 proteins are bona-fide constituents of the Arabidopsis DUBm should be addressed in future studies.

Subcellular localization of DUBm proteins in *Arabidopsis*.

Our previous data showed that SGF11, UBP22 and ENY2 physically interact in plant cell nuclei

and are the likely constituents of the Arabidopsis DUBm. To determine whether these proteins localize in additional cell compartments, we investigated their subcellular localization in *Arabidopsis* cells. To this aim, transgenic lines overexpressing GFP fusions to SGF11, ENY2 and UBP22 were generated and analyzed by fluorescent confocal microscopy. As shown in Figure 15, all fusions were detected exclusively in the nuclei, which is in agreement with a major role of the DUBm in chromatin remodeling by H2B deubiquitination.

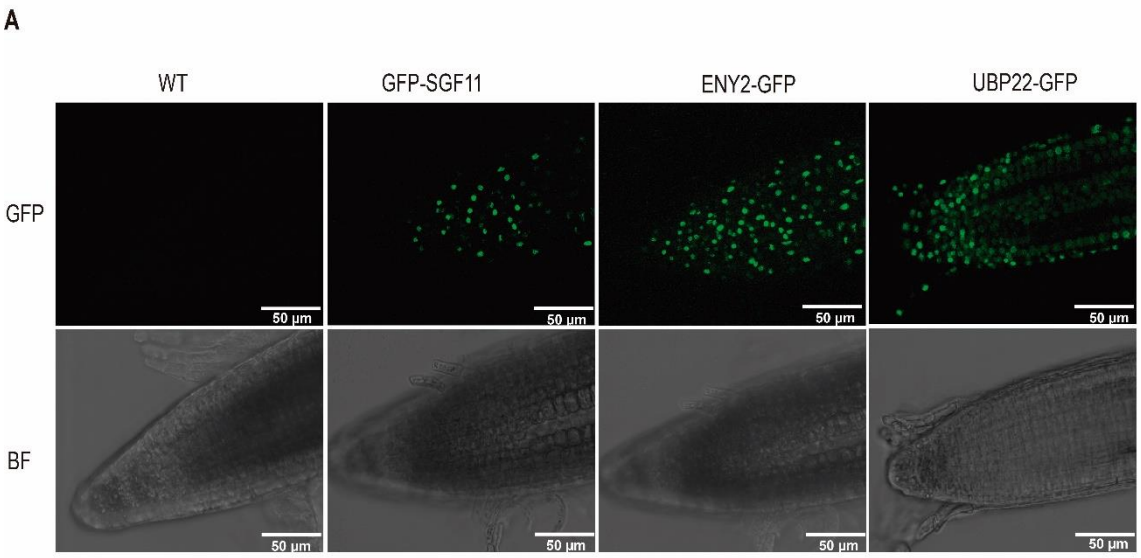


Figure 15. GFP fusions to DUBm components localize in nuclei. A) Images of roots of GFP-SGF11, GFP-ENY2, GFP-UBP22 expressing plants. In all cases 5 day-old seedlings were analyzed by fluorescent confocal microscopy. Bar=50 µm. All images are shown at the same magnification.

DUBm components and DDA1 localize in euchromatic regions.

The combinatorial association of H2Bub distribution along *Arabidopsis* genomic sequences has been recently analyzed. These epigenomic studies suggested that H2Bub marks associate with euchromatic regions where gene activation occurs (Minsky *et al*., 2011). To determine whether the DUBm components colocalize with H2Bub-enriched regions, epifluorescence imaging of DAP-stained nuclei from Arabidopsis plants expressing GFP fusions to SGF11, UBP22, and ENY2 was performed. As we previously found physical interaction between DDA1 and SGF11, DDA1-GFP expressing plants were also analyzed. In agreement with the distribution of H2Bub at the coding region of expressed genes in *Arabidopsis*,, SGF11, UBP22 and DDA1 fusions to GFP displayed euchromatin localization, However, in the case of ENY2-GFP fusion both eu- and heterochromatin localization was observed (Figure 16; results obtained in collaboration with Dr. Fredy Barneche´s group).

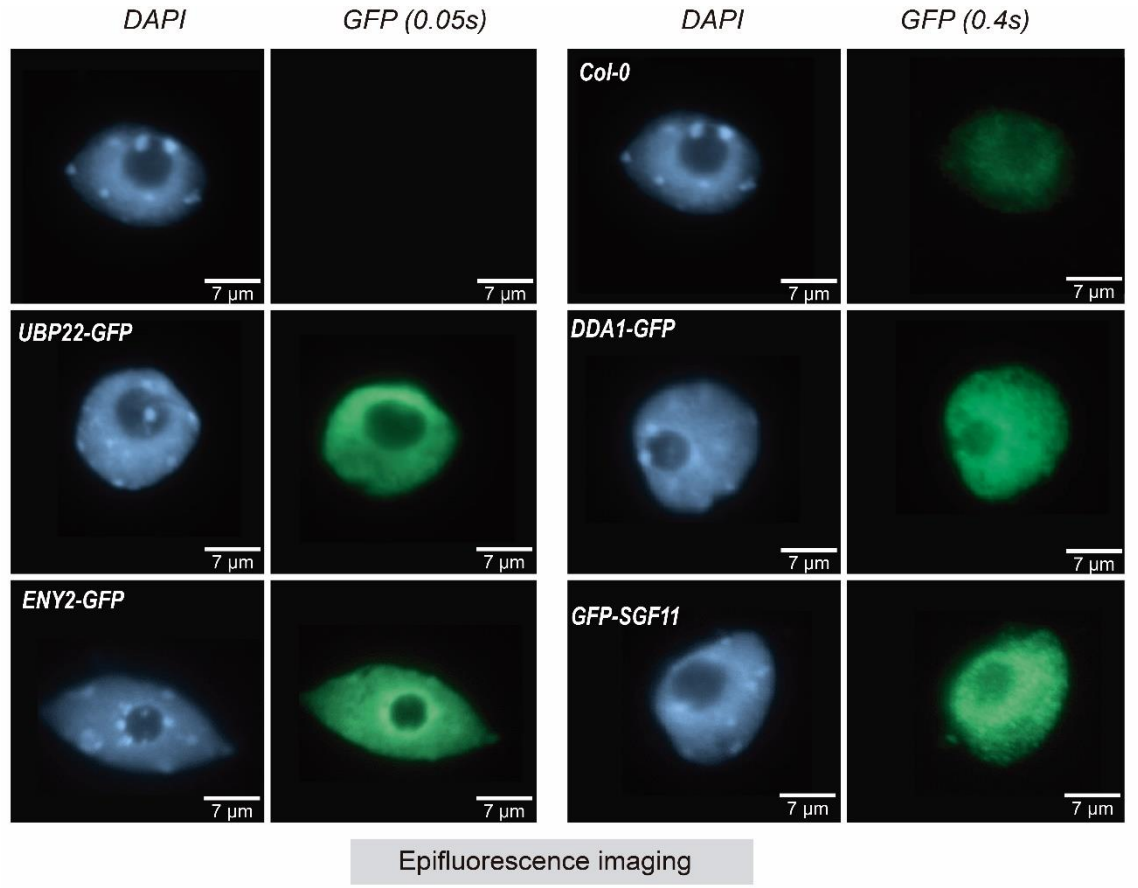


Figure 16. Epifluorescence microscopy analysis of the nuclear localization of DUBm components. Epifluorescence images of representative DAPI-stained nuclei from wild-type Col-0, and DDA1-GFP, GFP-SGF11, ENY2-GFP and UBP22-GFP expressing plants. Bar=7 µm. All images are shown at the same magnification.

Arabidopsis DUBm components mediate H2B deubiquitination.

In yeast, it has been shown that the DUBm specifically removes the ubiquitin moiety from H2Bub1 (Henry *et al*., 2003). To determine whether SGF11 and UBP22 are functional homologues of yeast Sgf11 and Ubp8 respectively, we examined the H2Bub1 pattern in wild-type and mutant plants (Figure 17A). By immunoblot analysis using an anti-H2Bub1 antibody, we found accumulation of H2Bub1 in nuclear protein extracts from *sgf11-1*, *sgf11-2* and *ubp22-1* mutants compared to the wild-type (Figure 17A and 17B). Together with our previous data, these results indicate that SGF11, ENY2 and UBP22 associate together forming the DUBm complex in *Arabidopsis,* which plays a major role in H2B deubiquitination.

In order to identify the limiting factor for the deubiquitinase activity of DUBm, H2Bub levels where analyzed in *Arabidopsis* lines overexpressing either SGF11 or UBP22 proteins fused to different tags (i.e. 10 repeats of the MYC epitope or GFP). We found that by overexpressing UBP22 the level of H2Bub was 50% reduced compared to the wild type, whereas SGF11 overexpression did not lead to a decrease in the H2Bub level (Figure 17C and 17D). Based on

these findings, we conclude that, according to its predicted enzymatic activity, UBP22 subunit is the protein subunit that limits the deubiquitinase activity of the DUBm in *Arabidopsis*.

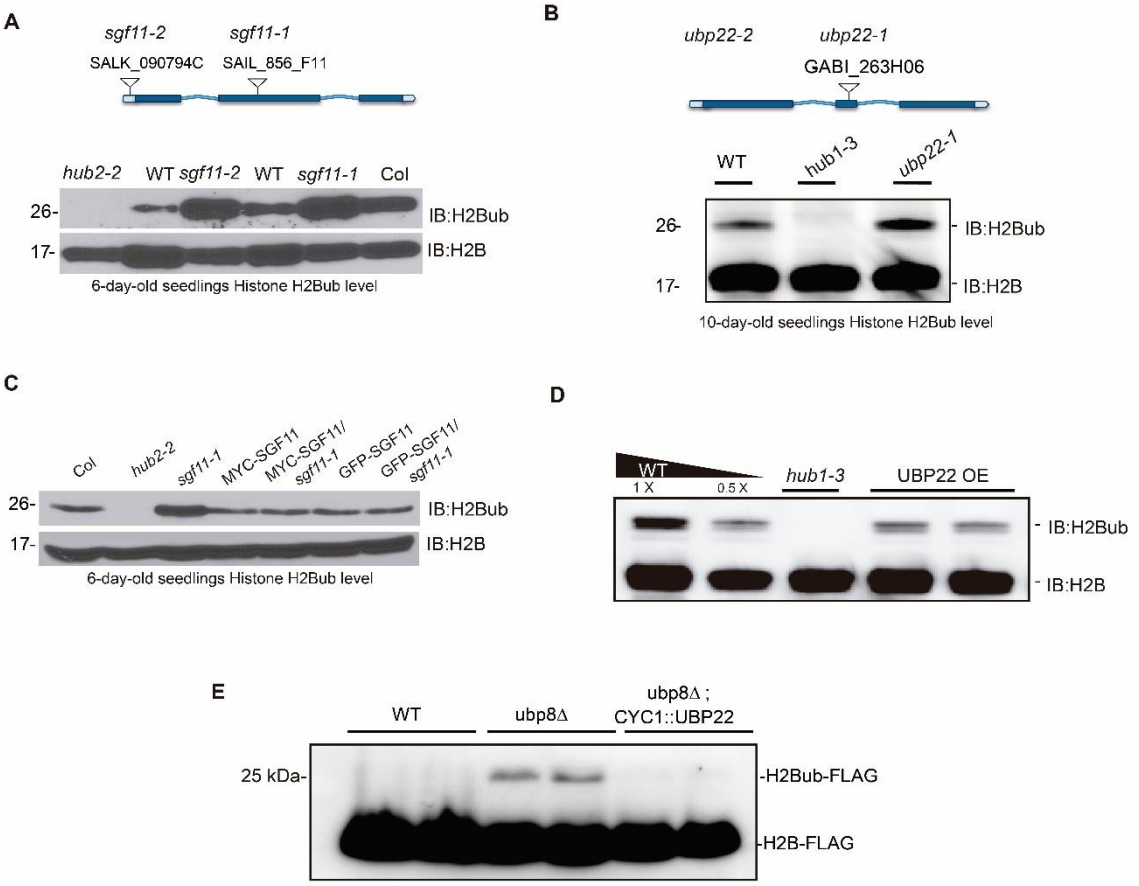


Figure 17. Analysis of the effect of altered expression of *SGF11* and *UBP22* in H2Bub1 levels. A) Immunodetection of H2Bub1 in wild-type and *sgf11* mutants The upper band corresponds to monoubiquitinated H2B (H2Bub1) detected using anti-H2Bub1 antibodies, whereas the lower band corresponds H2B detected by anti-H2B. B) Immunodetection of H2Bub1 in wild-type and *ubp22-1* mutants. Both H2B and H2Bub1 isoforms were detected using anti-H2B antibodies. C) Functional complementation assay of *sgf11-1* mutant defects in H2Bub1 deubiquitination in plants that overexpress either MYC-SGF11 or GFP-SGF11. D) Overexpression of UBP22 reduces H2Bub1 levels. Immunodetection was carried out as in (B). E) *Arabidopsis* UBP22 displays H2Bub1 deubiquitinase activity. Expression of *Arabidopsis UBP22* driven by the *CYC1* promoter in a yeast *ubp8*Δ strains complements the mutant defect by restoring H2Bub1 levels to those of the wild-type strain. All strains expressed Flag-tagged H2B. Immunoblots of cell lysates were performed using an anti-Flag antibody to detect both H2B isoforms.

These experiments also helped us to test the functionality of SGF11 and UBP22 fusions used in our studies since all constructs tested were able to complement the molecular defects observed in the corresponding mutant lines (*sgf11-1* and *ubp22-1*, respectively) with regard to H2B deubiquitination (Figure 17C).

Finally, we aimed to determine whether UBP22 displays H2BUb deubiquitinase activity itself. For this, we took advantage of available yeast *ubp8*Δ strains in which the H2Bub deubiquitination mediated by DUBm is severely impaired as shown by increased accumulation of H2Bub compared to wild-type controls (Richard *et al*., 2005; Figure 17E). Using immunoblots

against Flag-tagged H2B, we observed that expression of *Arabidopsis* UBP22 under the control of the CYC1 promoter restored the H2Bub levels in *ubp8*Δ yeast cells to those of wild-type yeast strains, indicating that UBP22 is a functional orthologue of Ubp8 (results obtained in collaboration with Fredy Barneche´s group) (Figure 17E).

Chapter III: *Arabidopsis* DUBm components are targets of the ubiquitin proteasome system.

Introduction.

As shown in chapters I and II, the DUBm module comprises at least three different subunits; UBP22, ENY2 and SGF11, and likely acts independently of the SAGA complex in *Arabidopsis*. In addition, our data show that CRL4-CDDD component DDA1 physically interacts with SGF11. Since CRL4-CDDD acts as an E3 ubiquitin ligase to target specific proteins for ubiquitination and proteasomal degradation, we hypothesize that SGF11, and possibly other DUBm components, are targets of the CRL4-CDDD activity. To test this idea, we analyzed whether SGF11, UBP22 and ENY2 are subjected to proteasomal destabilization depending on the luminic conditions and whether CRL4-CDDD plays a role in this process.

DUBm components are degraded by the proteasome preferentially in the dark.

To analyze the pattern of accumulation of DUBm components, we first obtained Arabidopsis transgenic lines that overexpress GFP fusions to each subunit: SGF11 (oeGFP-SGF11), UBP22 (oeUBP22-GFP) and ENY2 (oeENY2-GFP). In the case of SGF11 and UBP22, transgenic plants were obtained in the corresponding mutant backgrounds (*sgf11-1* and *ubp22-1*) to avoid any interference with the endogenous proteins. However, in the case of ENY2, for which no mutant was available, oeENY2-GFP plants were generated in the wild-type background. Independent transgenic lines showing highly detectable levels of each construct were selected. As well, in the case of oeGFP-SGF11 and oeUBP22-GFP lines, mutant complementation was confirmed, therefore probing the functionality of these GFP fusions (See Methods, Figure 6; Figure 17C and D). Next, we analyzed the protein levels for each construct under both light and dark conditions. As shown in figure 18, all DUBm protein fusions displayed higher protein levels when seedlings were grown under continuous light conditions compared to those grown in complete darkness, suggesting that darkness promotes DUBm proteins degradation. Additionally, the involvement of the proteasome in the accumulation of all three DUBm subunits was also examined by treating light- or dark-grown seedlings with proteasome inhibitor MG132 before harvesting them. As a result, clear accumulation of GFP fusions for SGF11, UBP22 and ENY2 was observed after 4 h of MG132 treatment under both light and dark conditions, although such an effect was more evident in the later case.

Thus, in dark-grown seedlings where only 9% SGF11, 8% UBP22 and 15% ENY2 GFP fusions

could be detected compared to fusions levels in light-grown plants, upon MG132 treatment, a very significant stabilization of all DUBm protein subunits was observed; reaching levels that in some cases were higher to those observed in transgenic plants grown under continuous light. (Figure 18 A, B and C).

The effective stabilization of DUBm proteins by MG132 indicates that 26S proteasome is responsible for the degradation of DUBm components that mainly occurs under dark conditions.

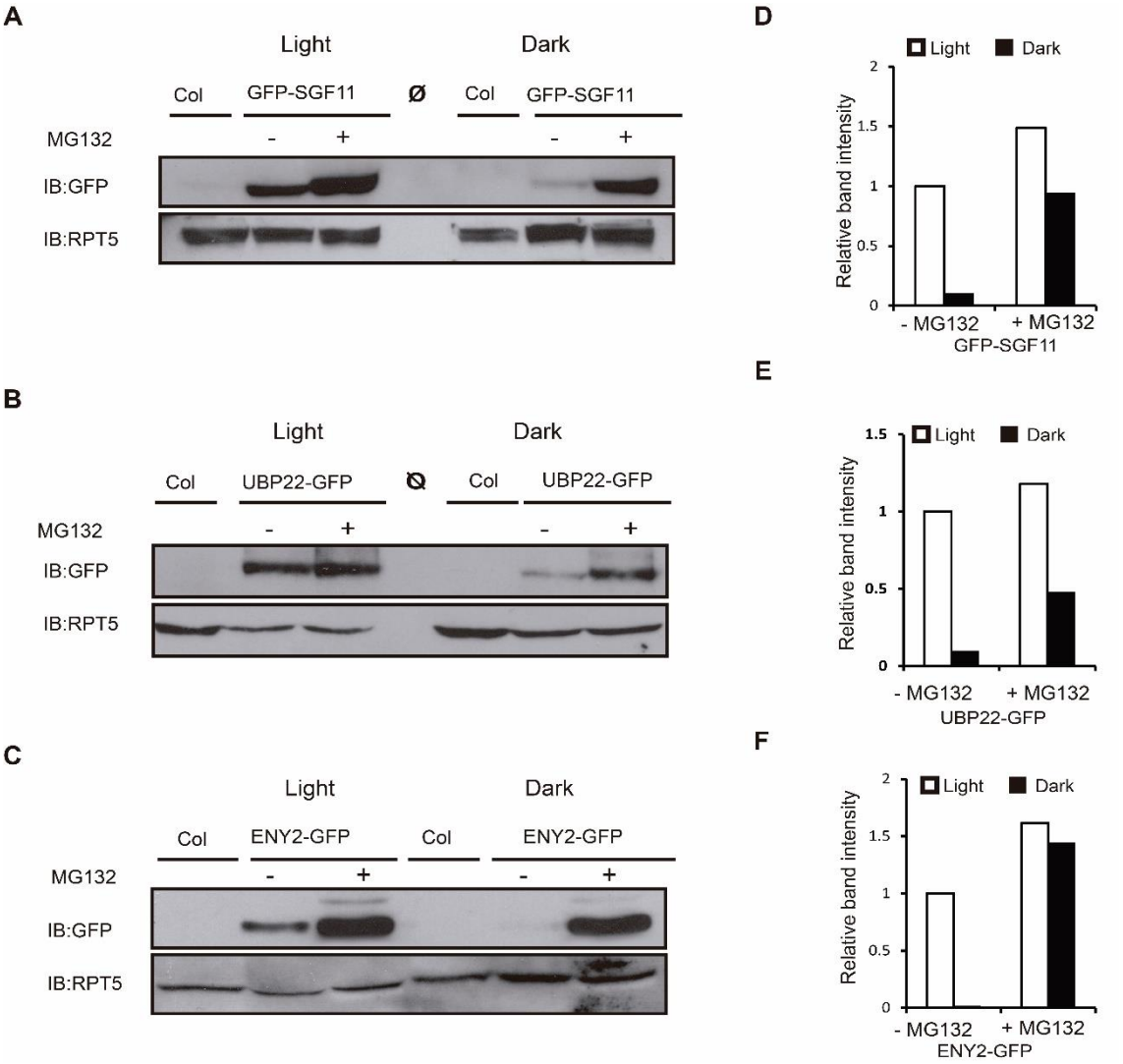


Figure 18: DUBm proteins are targeted for degradation by the proteasome mainly under dark conditions. A, B and C) Immunoblot analysis of the relative abundance of DUBm protein fusions to GFP in (MYC-SGF11, UBP22-GFP and ENY2-GFP) 6 day-old-seedlings grown under continuous light or dark conditions. Prior to plant harvesting, seedlings were treated (+) or not (-) with 50 µM MG132 for 4h. D, E and F) Band intensity analysis of protein fusions shown in A, B and C. RPT5 prtein was used as loading control to normalize protein levels. Results are shown relative to protein levels for each fusion under light conditions and in the absence of MG132. The analysis was performed using the ImageJ software.

DET1 promotes SGF11 protein degradation upon light to dark transition.

The fact that DUBm subunits are degraded in darkness together with the physical interaction between SGF11 and DDA1 led us to hypothesize that CRL4-CDDD E3 ligases could be mediating DUBm destabilization. To test this idea, we selected SGF11 as a representative member of the DUBm complex and checked the effect of altered function of CRL4-CDDD on its protein accumulation pattern. Since *dda1* loss of function mutants are sterile and do not set seeds, their use was precluded (Irigoyen *et al*., unpublished). Thus, we took advantage of the availability of weak Arabidopsis mutants for other CDDD components. This is the case of *det1-1* mutants, which express 1% *DET1* transcript levels compared to the wild-type, representing a partial loss of function allele (note that null *det1* mutants are embryolethal). Thus, we introgressed the functional MYC-SGF11 construct (Figure 17C) into the *det1-1* background to directly compare MYC-SGF11 protein abundance in wild-type (oeMYC-SGF11/WT) and *det1-1* (oeMYC-SGF11/*det1-1*) plants under light and dark conditions (Figure 19A and B).

Similar to our previous results (Figure 18), MYC-SGF11 accumulation was reduced under dark compared to light conditions oeMYC-SGF11/WT plants and MG132 treatment led to protein fusion stabilization (Figure 19A). However, similar MYC-SGF11 protein levels were found in dark- and light-grown oeMYC-SGF11/*det1-1* seedlings in the absence of MG132 (Figure 19B), suggesting that DET1 is required for the selective degradation of SGF11 in the dark. To determine whether the differences observed in MYC-SGF11 levels were due to altered expression of the transgene or to a posttranscriptional regulatory mechanism, the levels of MYC-SGF11 trascripts were analyzed by RT-qPCR in both oeMYC-SGF11/WT and oeMYC-SGF11/*det1-1* grown under continuous light and dark conditions. The results obtained indicated that changes in MYC-SGF11 protein levels between WT and det1-1 backgrounds under different luminic conditions do not correlate with those in transgene expression, indicating that MYC (Figure 19B). Thus, we concluded that MYC-SGF11 accumulation is subjected to posttranscriptional control, likely CRL4-CDDD-mediated ubiquitination and proteasomal degradation.

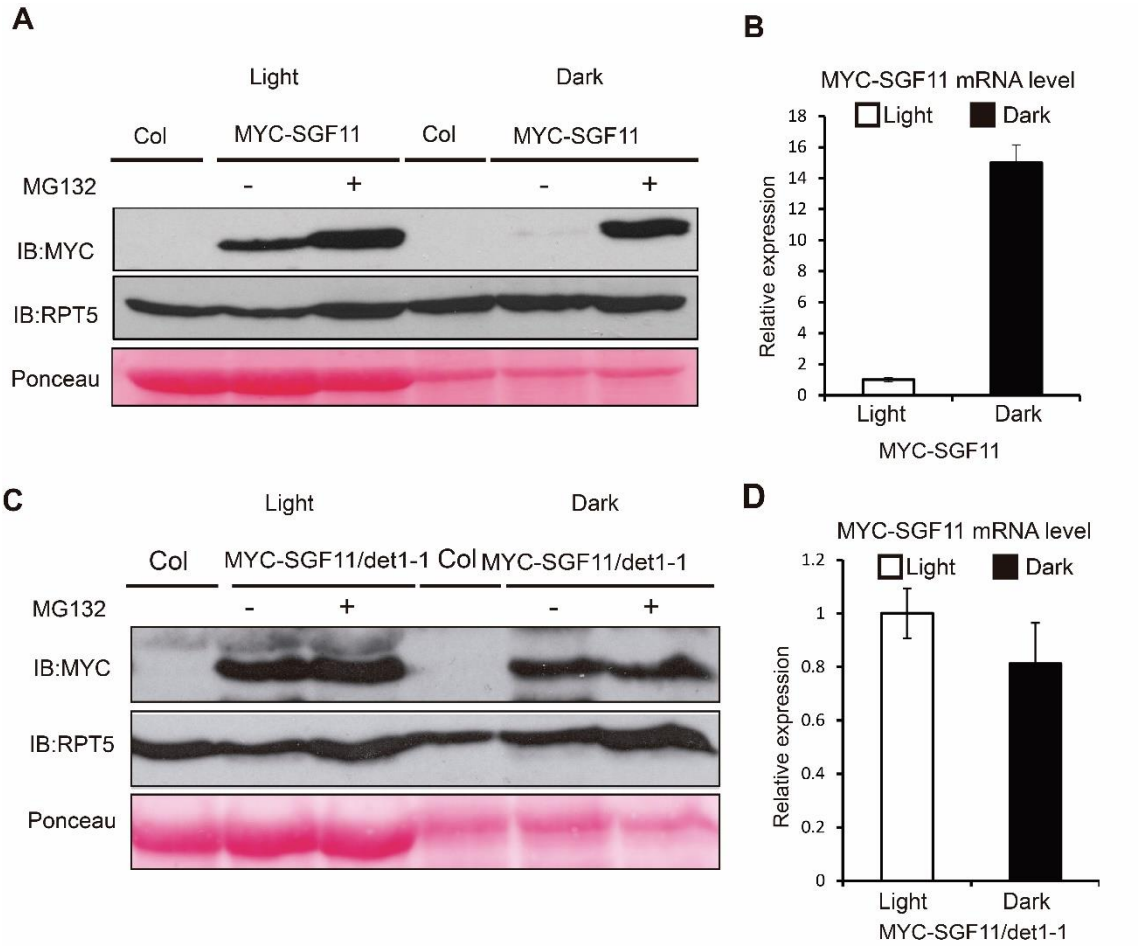


Figure 19: DET1 mediates SGF11 degradation in the dark. A and C) Analysis of the abundance of MYC-SGF11 fusion in 6 day-old oeMYC-SGF11/WT and oe-MYC-SGF11/*det1-1* seedlings grown under dark and light conditions. Prior to plant harvesting, seedlings were treated (+) or not (-) with 50 µM MG132 for 4h. MYC-SGF11 was detected using anti-MYC antibodies. Anti-RPT5 was used as loading control as well as Ponceau staining. B and C) Analysis of *MYC-SGF11* mRNA expression level by RT-qPCR in plants used in A and C. The data are the average of three biological replicates with two technical replicates each of them. Error bars represent the standard deviation.

As shown by MG132 treatments (Figures 18 and 19), MYC-SGF11 destabilization by the proteasome occurs mainly under dark conditions although there is some effect also in the light. We aimed to analyze the kinetics of MYC-SGF11 destabilization first by using protein synthesis inhibitor cycloheximide (CLX) during a time-course study. For this, we used oeMYC-SGF11/WT and oeMYC-SGF11/*det1-1* seedlings grown under continuous light or dark conditions treating them with CLX for different periods of time (1, 2, 3 and 4 h).

As a result (Figure 20A), we found that MYC-SGF11 protein levels remain stable up to 4h after CLX treatment under constant light conditions in both WT and *det1-1* backgrounds, indicating that SGF11 stability is not severely compromised under light conditions but it might require a switch in the luminic conditions (i.e. lights off) to trigger SGF11 destabilization. This effect might require DET1 function as MYC-SGF11 abundance remained constant in dark-grown *det1-*

1 seedlings (Figure 20B).

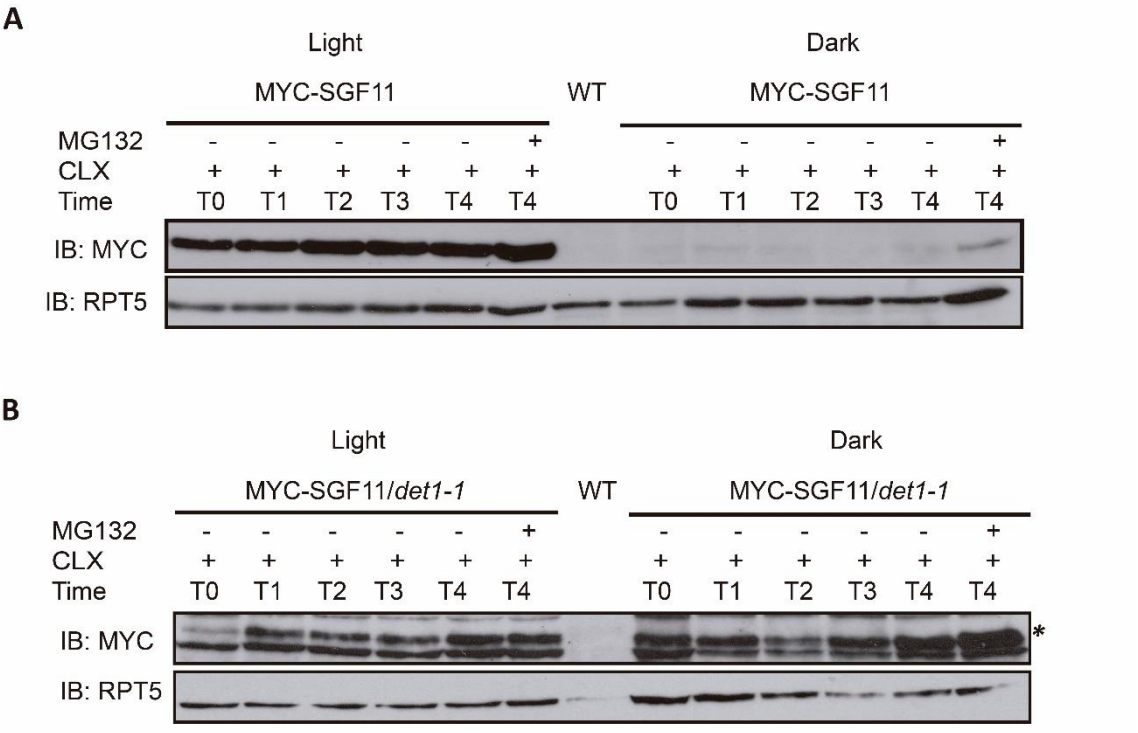


Figure 20: Analysis of the kinetics of MYC-SGF11 destabilization . A and B) Time-course assays upon treatment with cycloheximide (CLX) of continuous light- or dark-grown oeMYC-SGF11/WT (A) and oeMYC-SGF11/*det1-1* seedlings (B). For immunoblots, 40 µg protein extracts were used from 6 day-old-seedlings grown under light or dark, treated prior to harvest with 50 µM cycloheximide (CLX) for 0, 1, 2, 3 and 4 hours in the presence (+) or absence (-) of 50 µM MG132. Anti-MYC was used to detect MYC-SGF11. Anti-RPT5 was used as loading control. The asterisk (*) indicates the position of MYC-SGF11 which is around 47-kDa.

To test these ideas, we examined MYC-SGF11 protein levels during light to dark and dark to light transitions. For this, plants overexpressing MYC-SGF11 in wild type or *det1-1* background were grown in light or dark for 6 days and then transferred to dark or light, respectively, for 1, 2 and 3 days. Immunoblots from wild type seedlings transferred from light to dark revealed that the abundance of MYC-SGF11 was significantly reduced after just one day in dark conditions compared to time zero. However, reduction in MYC-SGF11 protein levels occurred at a much lower rate in the *det1-1* background upon transference to dark (Figure 21A and 21B). These results support the notion that DET1, and likely the whole CRL4-CDDD complex, mediate destabilization of SGF11 when lights are off.

By contrast, in seedlings shifted from dark to light, MYC-SGF11 accumulated gradually after 2 days in wild type seedlings. Surprisingly, SGF11 protein levels in *det1-1* mutant were reduced after one day in seedlings transferred from dark to light and remained constant, suggesting than additional regulatory mechanisms may act to fine tune SGF11 accumulation upon

transition from dark to light conditions (Figure 21).

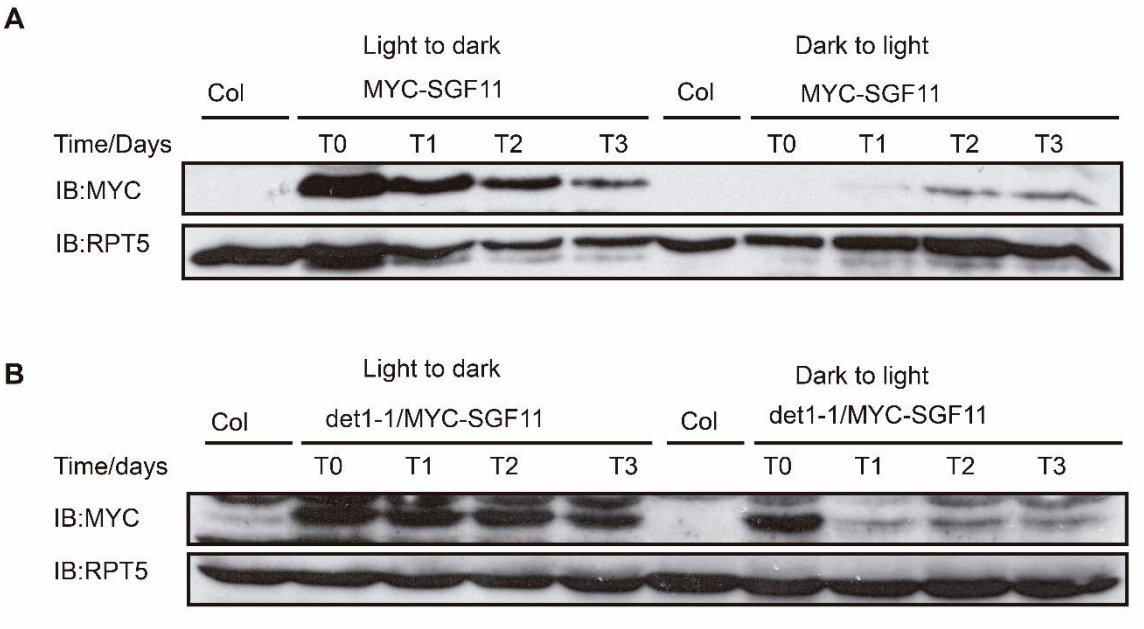


Figure 21. SGF11 degradation is controlled by the light conditions in a DET1 dependent manner. A) Immunoblot analysis of Arabidopsis seedlings expressing MYC-SGF11 in wild type or in *det1-1* background. Seedlings grown in dark or light for 6 days were shifted to light or dark, respectively, for 0, 1, 2 and 3 days. MYC-SGF11 was detected using anti-MYC antibodies. Anti-RPT5 t was used as a loading control.

SGF11 is a target of polyubiquitination.

Protein levels of all three DUBm subunits increased upon MG132 treatment, which indicates involvement of the proteasome in their degradation. Protein recognition by the proteasome occurs by its interaction with polyubiquitin chains (usually linked by their K11 or K48) that are conjugated to protein targets by the ubiquitination machinery. Based on this fact, we tested whether SGF11 is a target of polyubiquitination and whether this process occurs mainly in the dark. To this aim, 6-day old seedlings overexpressing MYC-SGF11 grown under continuous light conditions were transferred to dark and treated overnight with MG132. Similar treatment was performed under light conditions as a control. Upon protein extraction, ubiquitin-conjugated proteins were recovered using commercially-available p62 resin (Wilkinson *et al*., 2001). Immunoblot analysis using anti-MYC antibody revealed the presence of high-molecular weight bands corresponding to polyubiquitinated isoforms of SGF11. Interestingly, the level of SGF11 polyubiquitination was higher in seedlings incubated in darkness with MG132 than those were treated in the light (Figure 22), further supporting the notion that SGF11 is polyubiquitinated and degraded when lights are off.

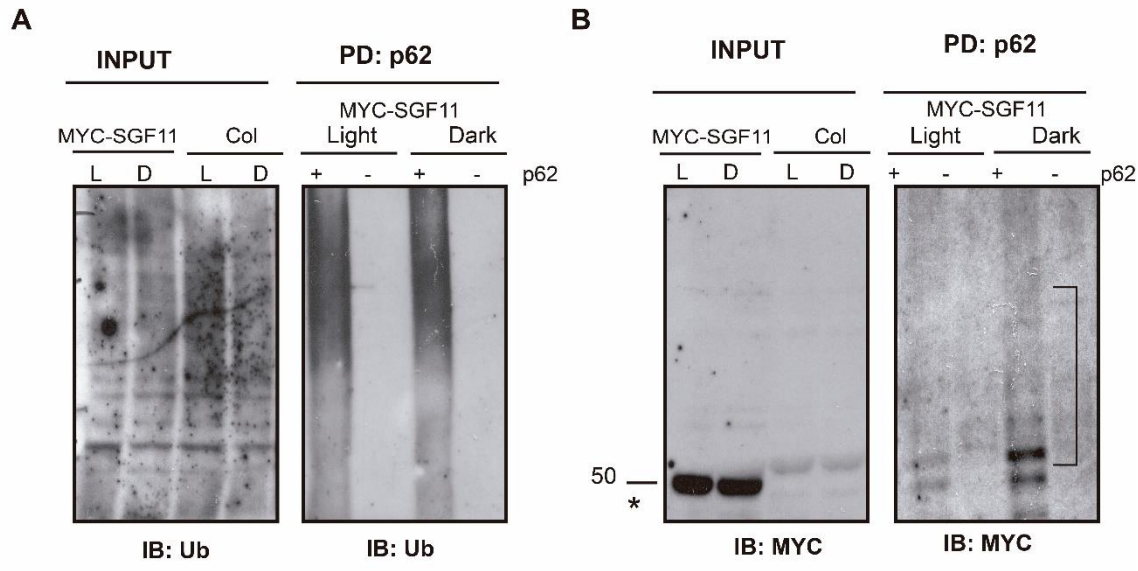


Figure 22: SGF11 is polybiquitinated mainly under dark conditions. A and B) Purification of polyubiquitinated MYC-SGF11. MYC-SGF11 protein extracts were incubated with P62 resin or with agarose resin as negative control. The anti-Ub antibody was used in (A) to detect total ubiquitinated proteins. Anti-MYC allows detection of MYC-SGF11 and its polyubiquitinated isoforms in (B). Col-0 wild type protein extracts were used as immunoblot control (Col). The asterisk (*) indicates the position of non-ubiquitinated MYC-SGF11 which is around 47-kDa. A bracket indicates the position of polyubiquitinated MYC-SGF11 (Ub(n)-MYC-SGF11).

Chapter IV: CDDD components modulate ubiquitinated H2B levels.

Introduction.

As it has been shown in chapter II, the Arabidopsis DUBm mediates H2B deubiquitination, similar to what it has been shown in other model organisms such as yeast or humans. As well, data described in chapter III indicated that DUBm components SGF11, UBP22 and ENY2 are degraded by the proteasome mainly under dark conditions. This activity is likely mediated by CRL4-CDDD E3 ligases, through direct recognition of SGF11 by DDA1. Thus, we hypothesized that, by controlling DUBm levels depending on the light conditions, CRL4-CDDD complexes may influence H2B ubiquitination. In fact, ubiquitination of H2B deposited at specific loci plays a role in the modulation of their responsiveness to light. Therefore, CRL4-CDDD E3 ligases might control the stability of DUBm to regulate specific gene expression according to changing light conditions. To test this model. we first determined whether altered function of CRL4-CDDD affects the abundance of ubiquitinated H2B (H2Bub).

DDA1 and DET1 function alters H2Bub levels.

In order to test the afore-mentioned model, immunoblot analysis of H2Bub protein were carried in weak (*ddb1a, det1-1,* and *cop10-4*) mutants or overexpression lines (oeDDA1-GFP and oeGFP-DET1) for CRL4-CDDD components available in our laboratory and compared to their respective controls. In these assays we also included *cop1-4* mutants, since COP1 has been shown to functionally associate to CRL4-CDDD E3 ligases (Lau and Deng, 2012).

Among all lines tested, alteration in H2bub level was only detected in *det1-1* single mutants and oeDDA1-GFP lines (Figure 23A and 23B). Thus, we observed increased H2Bub levels in oeDDA1-GFP seedlings compared to the wild type. In contrast, a very significant decrease in H2Bub levels,was observed in *det1-1* mutants compared to the wild type (Figure 23B). No significant changes in H2Bub levels were observed in the rest of CDDD-related lines, although this could be due to the fact that *ddb1a* and *cop10-4* mutants represent very weak mutant alleles. A slight increase in H2B ubiquitination could be appreciated in *cop1-4* mutants although protein loading was higher in that sample and this effect could not be reproduced in other biological replicates. These results imply a potential regulatory role of CRL4-CDDD in H2B ubiquitination status by mediating the degradation of DUBm components in *Arabidopsis.*

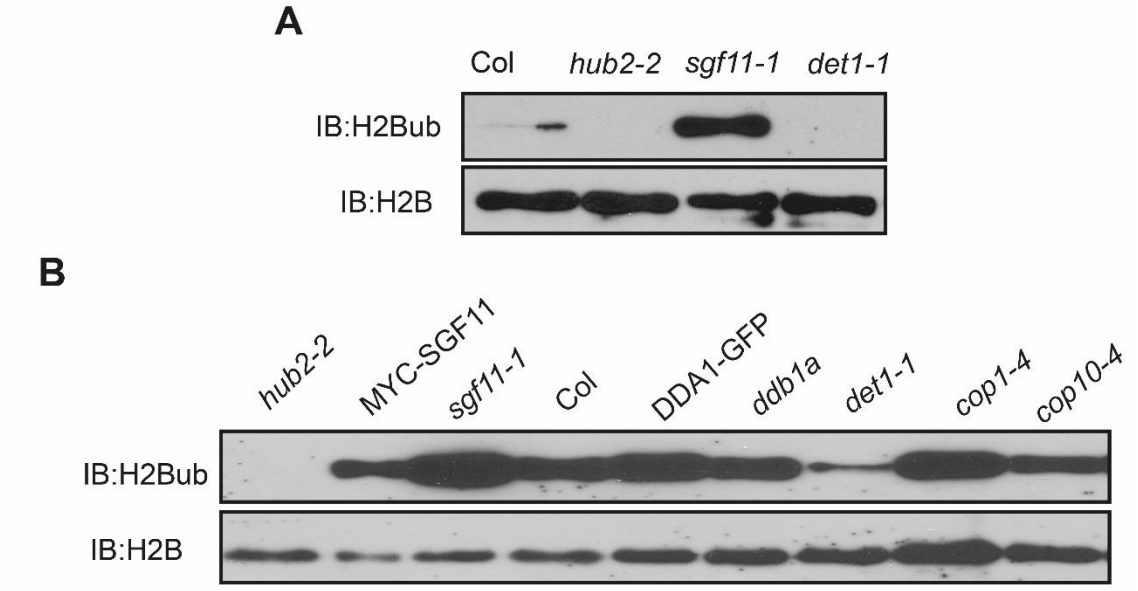


Figure 23. CDDD components are altering Histone H2B monoubiquitination levels. A) Detection of monoubiquitinated H2B (H2Bub) in wild-type and *sgf11-1* and *det1-1* mutant plants. The upper band corresponded to, H2Bub and the lower band was H2B used as loading control. B) Detection of H2Bub in wild-type and oeDDA1-GFP and *ddb1a, det1-1, cop1-4* and *cop10-4* mutant plants. Anti-H2Bub and anti-H2B antibodies were used for immunoblotting.

DET1 and DDA1 function affects specific H3 methylation marks.

We further investigated whether modulation of H2Bub levels by DDA1 and DET1 is extended to other histone modifications. For this, immunoblot analysis of H2Bub as well as H3K36me3 and H3K4me3 marks was analyzed in lines with reduced DET1 or increased DDA1 function. As a result, we observed that H3K36me3 accumulation was lower in *det1-1* plants than in the wild type, which correlated with reduced H2Bub levels. However, no changes in H3K36me3 levels were detected in other lines tested (Figure 24).

In parallel to higher accumulation of H2Bub in oeDDA1-GFP seedlings, the level of H3K4me3 was increased in this line (Figure 24, third panel). Our results suggest that, by altering H2Bub levels, DDA1 and DET1 influence other histone modifications. Interestingly, although H2Bub level was increased in *sgf11-1* seedlings, no obvious effect on global levels of H3K36me3 or H3K4me3 was observed in these mutants (Figure 24C).

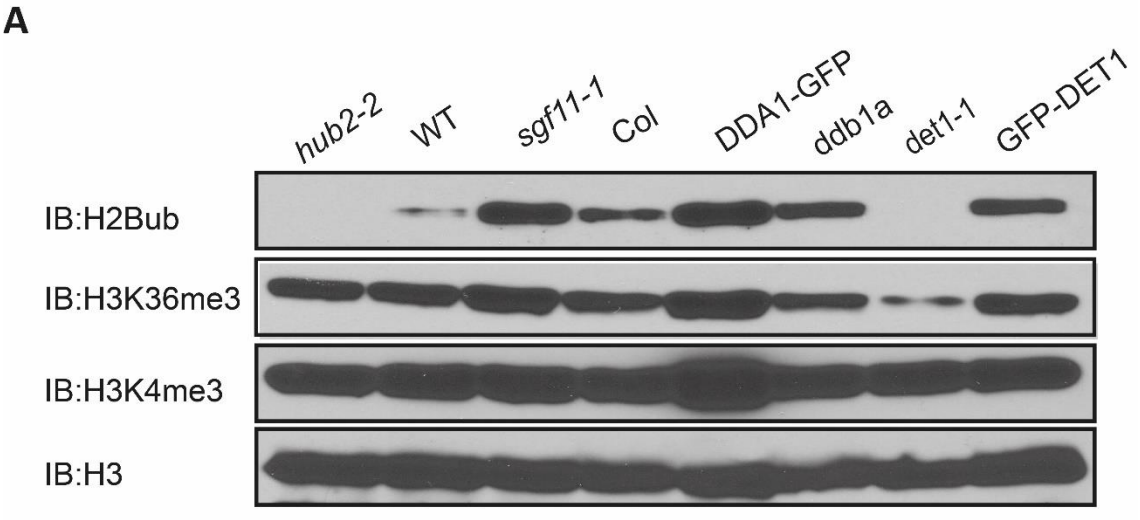


Figure 24. Analysis of global levels of specific modified histones in lines with altered DDA1 and DET1 function. A) Immunoblot analysis of global levels of monoubiquitinated H2B (H2Bub) and methylated H3 in lines with altered function of the CDDD. Histones were extracted from 6 day-old seedlings grown under continuous light conditions. *hub2-2* was used as a control for H2B ubiquitination. Whereas *sgf11-1* mutants were used as positive control for increased H2B ubiquitination. Anti-H3 was used as a loading control.

Additive effect of sgf11-1 and det1-1 mutations in H2Bub accumulation.

Our previous data indicated that destabilization of DUBm proteins mainly occurs under dark condition and is likely mediated by CRL4-CDDD E3 ligases. The fact that an opposite pattern of H2Bub accumulation was observed in oeDDA1-GFP plants and *det1-1* mutants supports this model. Thus, SGF11 levels should be reduced in oeDDA1-GFP plants (leading to H2Bub accumulation) and increased in *det1-1* mutants (promoting H2B deubiquitination). According to this model DDA1 and DET1 should be epistatic to SGF11 in the control of H2Bub accumulation . In order to test this idea, we obtained *sgf11-1 det1-1* double homozygous mutants (by crossing *sgf11-1* with *det1-1* plants) and examined their H2Bub levels compared to those in *sgf11-1* and *det1-1* single mutants under light and dark conditions. We found that *sgf11-1 det1-1* double mutants accumulate more H2Bub than single *det1-1* mutants but further less than *sgf11-1* mutants under both conditions, indicating an additive effect of both mutations on H2Bub accumulation (Figure 25). However, these results could also be explained by a role of DET1 as a regulator of the activity of additional ubiquitin proteases acting on H2Bub.

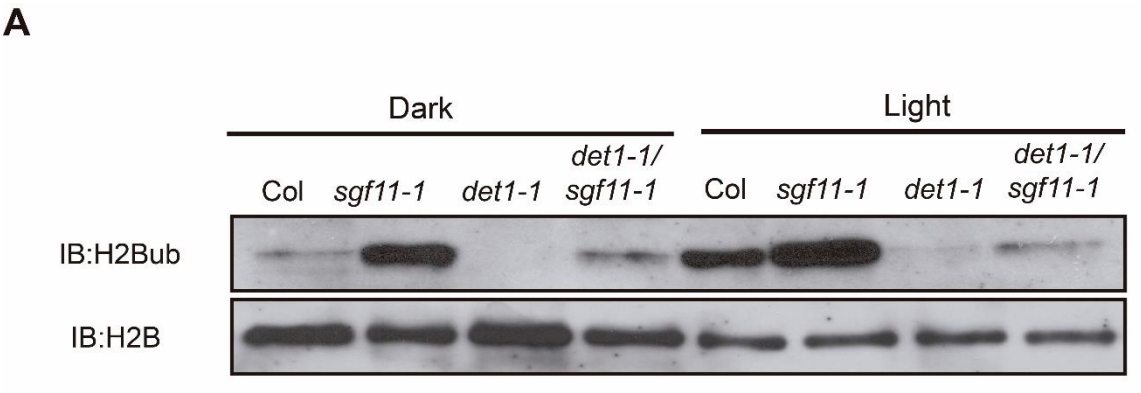


Figure 25. Additive effect of *sgf11-1* and *det1-1* mutation in H2Bub accumulation. A) Immunoblot analysis of total (H2B) and ubiquitinated (H2Bub1) histone H2B under light and dark condition. Wild-type (Col), *sgf11-1* and *det1-1* single mutants and *det1-1 sgf11-1* double mutants grown under continuous light or dark conditions were used. 16 kDa band corresponding to unmodified H2B was used as loading control.

Chapter V: DUBm components SGF11 and ENY2 facilitate negative control of photomorphogenesis by CRL4-CDDD.

Introduction:

Our previous results showed that CRL4-CDDD E3 ligases promote targeted destabilization of SGF11, and likely other DUBm components. In this way, CRL4-CDDD complexes may influence the ubiquitination status of H2B, and therefore transcriptional expression, in genes that respond to light to negatively regulate photomorphogenesis. According to this idea, CRL4-CDDD and DUBm should play opposite roles in the control of light responses. We aimed to test this idea by using a genetic approach. Thus, we took advantage on the availability of mutants and overexpression lines for DUBm components, previously described in this study; so we studied their effect in the *det1-1* mutant background under different light treatments. Thus, *sgf11-1 det1-1* double mutants and oeMYC-SGF11 transgenic plants in the *det1-1* background were obtained. Similarly, we generated *ubp22-1 det1-1* double mutants and oeUBP22-GFP plants in the *det1-1* background. As described as follows, we characterized diverse photomorphogenic responses in those lines and compared them to those in single and wild type controls.

SGF11 and UBP22 are positive regulators of DET1 function in the dark.

Using the afore-mentioned SGF11/DET1 lines, we characterized their behavior under different light conditions, including continuous darkness, light and low light treatments. As shown in Figure 26, when plants were cultivated in the dark, hypocotyl length in *sgf11-1* mutants and oeMYC-SGF11/WT plants did not differ from that in the wild type control. However, we observed shorter hypocotyls in *sgf11-1 det1-1* mutants compared to the *det1-1* single mutant, indicating a synergistic effect of *sgf11-1* and *det1-1* mutations in hypocotyl growth. By contrast, overexpression of MYC-SGF11 attenuated the defects of *det1-1* mutants, as oeMYC-SGF11/*det1-1* plants displayed significantly longer hypocotyls that *det1-1* plants in the dark. In the case of seedlings grown in continuous light, *det1-1* mutants display smaller cotyledons with decreased chlorophyll and increased anthocyanin levels compared to the wild-type. Interestingly, cotyledon width and anthocyanin content of *sgf11-1 det1-1* double mutants were decreased and increased, respectively, relative to *det1-1* single mutants (Figure 26). However, plants that overexpressed oeMYC-SGF11 in the *det1-1* background did not differ significantly from *det1-1* plants with respect to any of these traits in the light

It has been previously shown that dim light helps to observe mild phenotypes in plants with defective light responses (Halliday *et al*., 2009). In order to test whether plants with altered SGF11 activity display photomorphogenic phenotypes and their genetic interaction with *DET1*, we cultivated our lines under low light conditions (see Methods). Under these conditions, *sgf11-1* mutants displayed shorter hypocotyls than wild type plants, whereas oeMYC-SGF11 seedlings exhibited a slightly longer hypocotyl compared to control plants (Figure 27A).

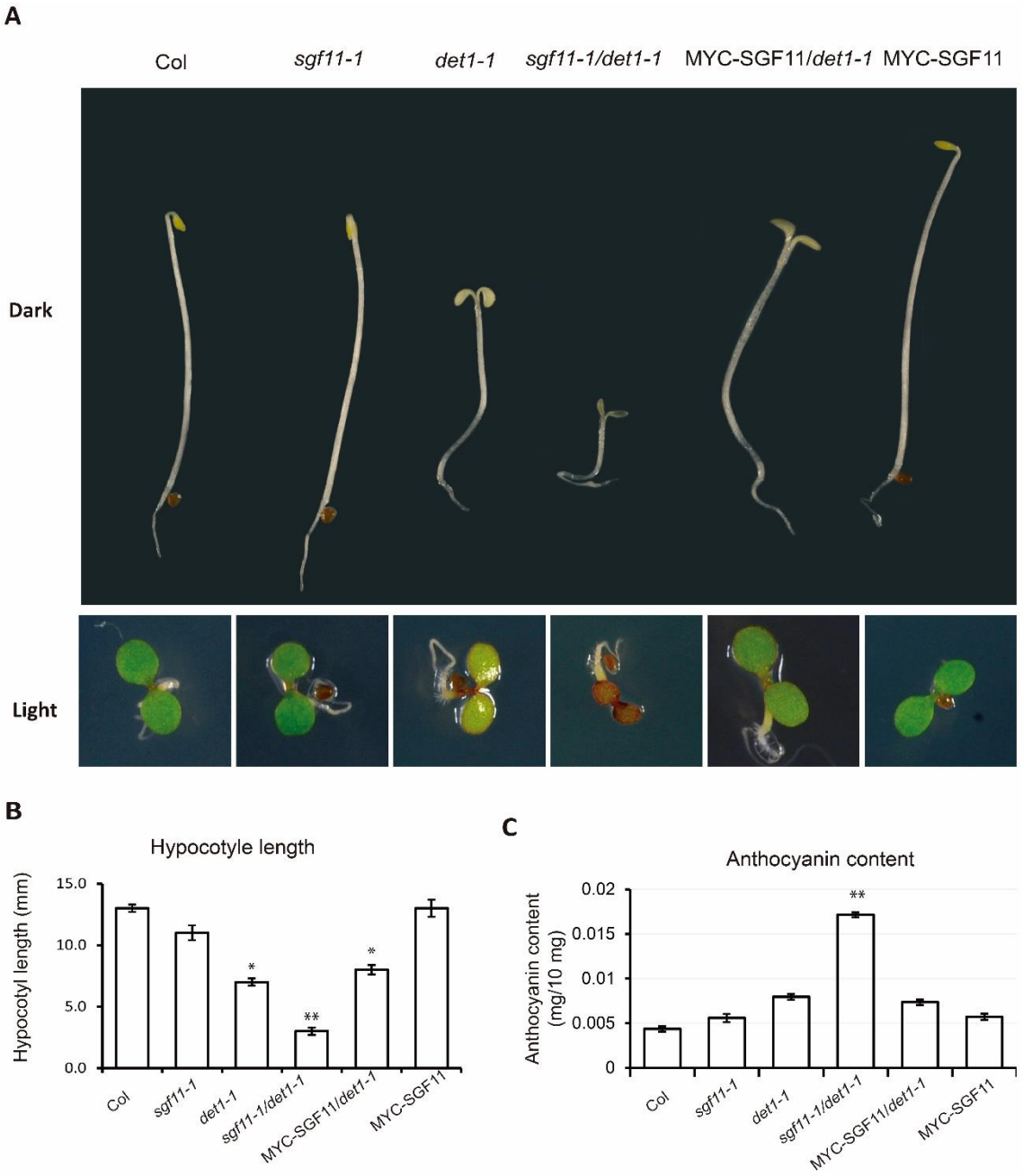


Figure 26. *sgf11-1* mutation aggravates the constitutive photomorphogenic phenotype of *det1-1* mutants in the dark. A) Images of 3 days-old seedlings corresponding to the wild type Col-0 (Col), *sgf11-1, hy5, det1-1*, *sgf11-1 det1-1,* oeMYC-SGF11/*det1-1* and oeMYC-SGF11/WT backgrounds grown under dark (upper panel) or light (lower panels) conditions. B) and C) Measurements of hypocotyl length (B) and anthocyanin content (C) of plants shown in (A) grown under continuous dark conditions for 3 days. The data indicate the mean values plus the SD from three independent experiments with 30 seedlings each per line. * $p < 0.05$, ** $p < 0.005$. n = 30. Error bars = ± S.D. (D).

However, there were no significant differences in the hypocotyl length between single *det1-1* and double *sgf11-1 det1-1* mutants or oeMYC-SGF11/*det1-1* plants (Figure 27). Altogether, our results indicate that SGF11 facilitates DET1 function as a negative regulator of photomorphogenesis and that this function might be particularly relevant when lights are off.

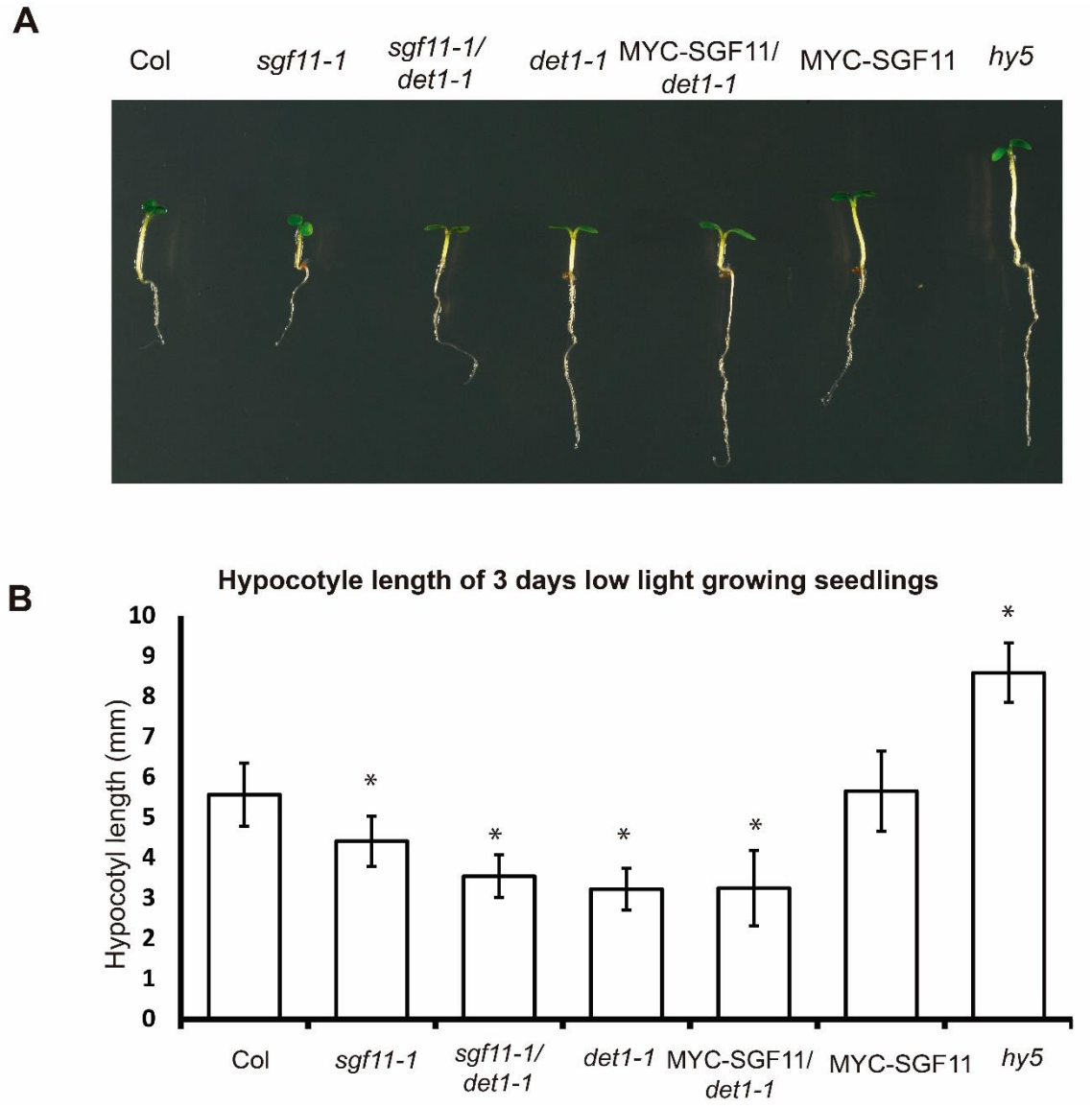


Figure 27. *sgf11-1* mutants exhibit short hypocotyls under continuous low light conditions. A) Images of 4 days-old seedlings corresponding to the wild type Col-0 (Col), *sgf11-1, hy5, det1-1*, *sgf11-1 det1-1*, oe MYC-SGF11/*det1-1* and oeMYC-SGF11/WT grown under low light conditions (800 lux; light conditions in other experiments was 4600 lux).. **B**) Measurements of hypocotyl length of plants shown in (A). * $p < 0.05$. n = 30. Error bars = ± S.D. (D).

To determine whether our findings on the genetic interaction between *SGF11* and *DET1* are extensive to other DUBm subunits, *ubp22-1 det1-1* double mutants were obtained and their

phenotypes were characterized in the dark. For this, hypocotyl length of 3-day old dark-grown *ubp22-1 det1-1* seedlings was measured and compared to wild type and single mutant controls. Similar to *sgf11-1 det1-1* plants, *ubp22-1 det1-1* double mutants exhibited shorter hypocotyls than *det1-1* single mutants in darkness (Figure 28B). Noteworthy, as previously observed for *sgf11-1* plants, dark-grown *ubp22-1* mutants did not differ in any aspects to wild type controls. These results indicate that the *ubp22-1* mutation also has a synergistic effect on *det1-1* photomorphogenic defects, although at a lesser extent than sgf11-1, since anthocyanin content or cotyledon width of ubp22-1 *det1-1* plants were as those in det1-1 plants (Figure 28C and data not shown).

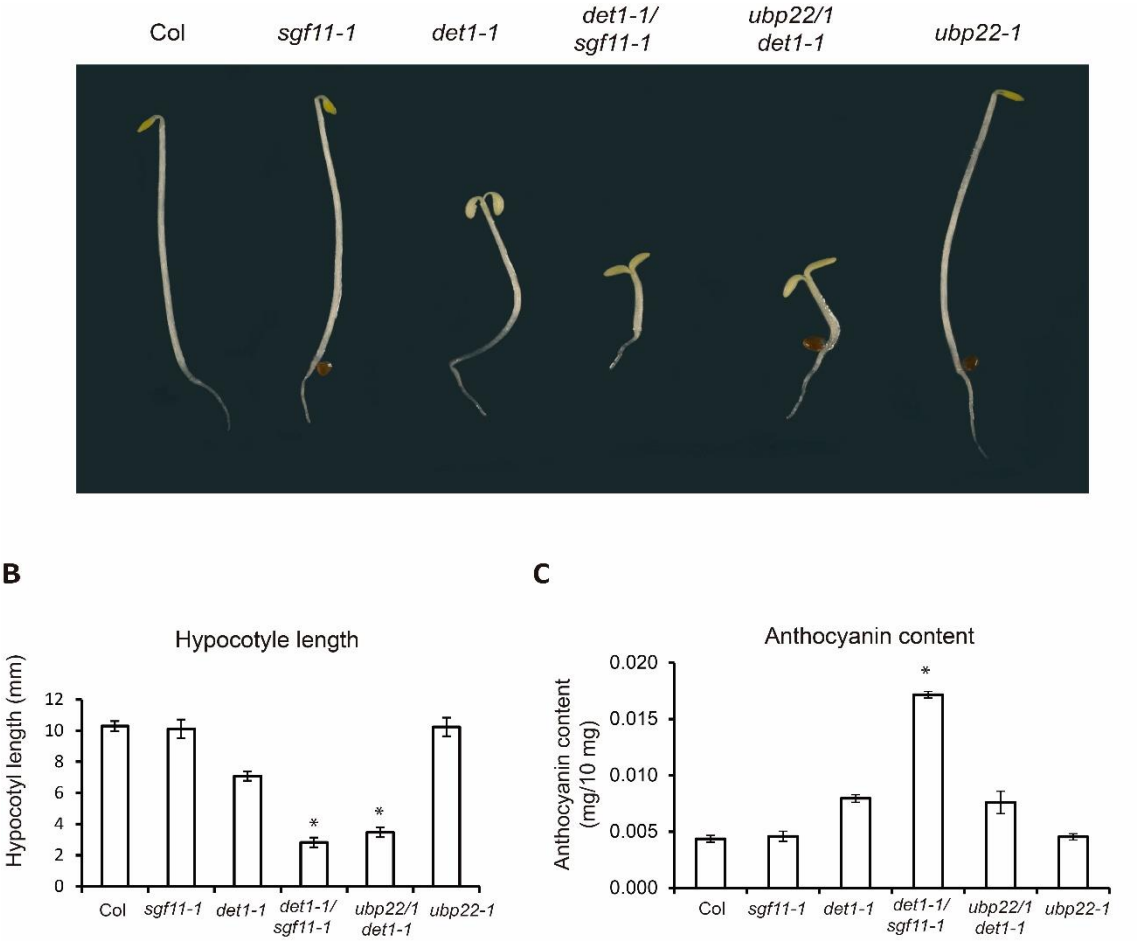


Figure 28. *ubp22-1* mutation aggravates the constitutive photomorphogenic phenotype of *det1-1* mutants in dark. A) Images of 3 days-old dark-grown seedlings corresponding to the wild type Col-0 (Col), *ubp22-1, det1-*, *det1-1 sgf11-1*, *det1-1 ubp22-1* double mutants and oeUBP22-GFP backgrounds. B) and C) Measurements of hypocotyl length (B) and anthocyanin content (C) of plants shown in (A) grown under continuous dark conditions for 3 days. * $p < 0.05$. n = 30. Error bars = ± S.D. (D).

Constitutive photomorphogenic phenotypes in lines with altered DUBm/CRL4-CDDD function correlate with their HY5 levels.

Transcription factor HY5 is a mayor positive regulator of light responses in plants. Indeed, it has been demonstrated, in most cases, that the constitutive detiolated phenotype of *cop/det/fus* mutants arises from a defect to effectively degrade HY5 in the dark. Based on this fact, we aimed to analyze whether constitutive photomorphogenic phenotypes in lines with altered DUBm/CRL4-CDDD function correlate with their HY5 levels. For this, HY5 protein abundance of 3 day-old light or dark grown seedlings corresponding to wild type Col-0 (Col), *sgf11-1*, *hy5*, *det1-1*, *sgf11-1 det1-1*, oe MYC-SGF11/*det1-1* and oeMYC-SGF11/WT grown, was analyzed by immunoblotting. Under light conditions, HY5 abundance did not differ significantly in any of the lines tested. By the contrary, as previously reported (Osterlund *et al*., 2000a and Osterlund *et al*., 2000b), HY5 abundance was increased in *det1-1* mutants cultivated in the dark. Strikingly, such an increase in HY5 abundance was enhanced or reduced in *sgf11-1 det1-1* double mutants or oeMYC-SGF11/*det1-1*, respectively, indicating that correlation exists between detiolation degree of those lines in the dark and their HY5 protein levels. In this regard, we observed a slight increase in HY5 abundance in *sgf11-1* single mutant compared to the wild type (Figure 29A).

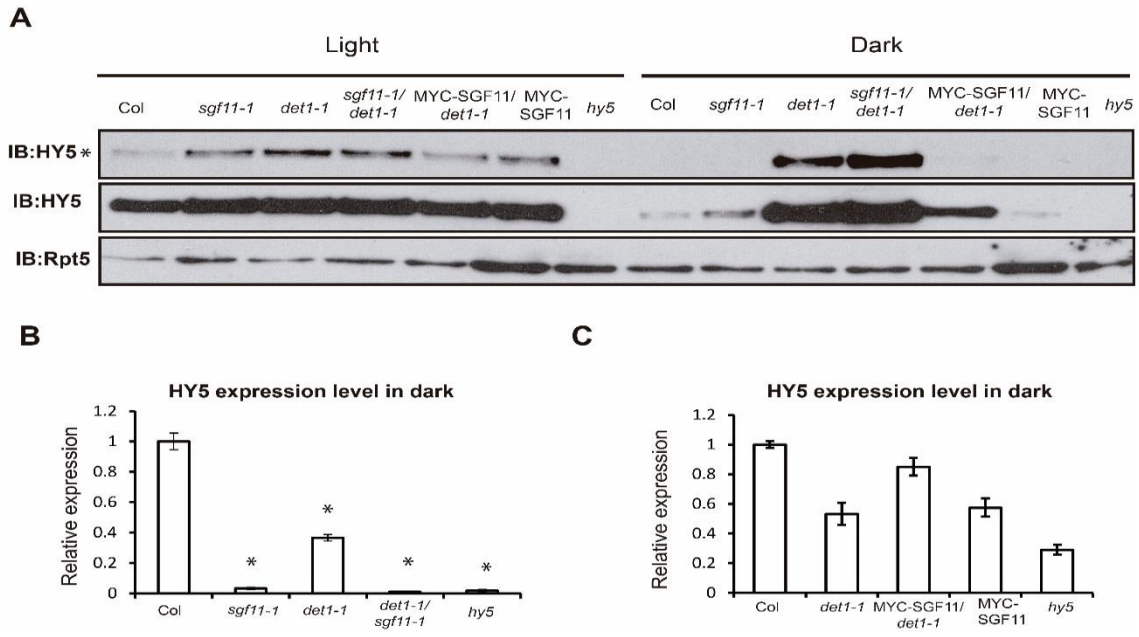


Figure 29. Analysis of HY5 protein abundance in mutant lines with altered DET1 and SGF11 function. A) Immunoblot analysis showing HY5 protein accumulation in light- and dark-grown seedlings corresponding to the wild type Col-0 (Col), *sgf11-1, hy5, det1-1*, *sgf11-1 det1-1,* oeMYC-SGF11/*det1-1* and oeMYC-SGF11/WT backgrounds. Protein extracts were prepared from 3 day-old seedlings grown under continuous light or dark conditions. A band of 15-kDa of HY5 was detected using anti-HY5 (Osterlund *et al*., 2000). Anti-RTP5 was used to detect a band of 49-kDa as loading control. B) and C) Relative expression level of *HY5* transcripts was analyzed by RT-q PCR in lines shown in (A) under dark conditions. * $p < 0.05$. n = 30. Error bars = ± S.D.

To determine whether altered accumulation of HY5 protein in those lines is due to changes in *HY5* transcript abundance or to a posttranscriptional regulatory mechanism (i.e. ubiquitination and targeted degradation), RT-qPCR analyses of *HY5* gene expression were carried out. As shown in Figure 29B and 29C, *HY5* mRNA levels were lower in *sgf11-1* and *sgf11-1 det1-1* mutants compared to other lines, under dark conditions, indicating that alteration in the accumulation of HY5 protein might due to a posttranscriptional regulatory mechanism. However, the decrease in HY5 protein displayed by dark-grown oeMYC-SGF11/*det1-1* plants did not correlate with the the level of *HY5* transcripts compared to single *det1-1* mutants (Figure 29B and 29C), suggesting that SGF11 overexpression ameliorates the defects of *det1-1* plants when promoting degradation of HY5. Altogether, these results indicate that SGF11 and DET1 act synergistically to promote HY5 destabilization under dark conditions, although additional effects on transcriptional activity of the *HY5* gene cannot be excluded.

DISCUSSION

Discussion.

The relevance of the Ubiquitin Proteasome System (UPS) as a key regulator of plant development in response to light has been extensively demonstrated. Among all UPS components, E3 ubiquitin ligase COP1, as part of CRL4 complexes in association with SPA proteins, displays a leading role in this process, acting as a well-known repressor of photomorphogenesis. Thus, COP1 targets for ubiquitination and proteasomal degradation numerous positive regulators of light signaling, spanning from light perception (i.e. phyA and CRY2) to transcriptional regulation (i.e. HY5, LAF1, HFR1, CO,…). COP1 function requires the activity of the COP9 signalosome (CSN), a 600 KDa complex that modulates assembly of $CRL4^{COP1\text{-}SPA}$ E3 ligases (by controlling the neddylation status of CUL4) and favors nuclear localization of COP1 (by an unknown mechanism). Indeed, complete loss of function of any component of $CRL4^{COP1\text{-}SPA}$ and CSN yields constitutively photomorphogenic plants that die during seedling stage. The COP10-DET1-DDB1-DDA1 (CDDD) complex is also known to facilitate the activity of the $CRL4^{COP1\text{-}SPA}$ complex, i.e. to promote degradation of transcription HY5, although the molecular basis of such activity has not been fully demonstrated. Thus, it has been proposed that COP10 enhances the activity of E3 ligases, likely including $CRL4^{COP1\text{-}SPA}$, to promote seedling etiolation under dark conditions. Within the CDDD complex, DET1 activity might be closely associated to chromatin remodeling and gene expression. Thus, DET1 is known to interact with non-acetylated tails of H2B and mediates transcriptional repression of specific genes. However, the precise molecular details on how DET1, and the whole CDDD complex, facilitates COP1-mediated repression of photomorphogenesis and the links with chromatin dynamics are unknown.

Chromatin remodeling activities are known to modulate gene expression in response to light. These include histone acetylation, mediated by SAGA complex component GCN5, and deacetylation, promoted by a set of histone deacetylases (i.e. HD1 and HD19), that induce and repress, respectively, transcription of light-responsive genes. H2B monoubiquitination (H2Bub) has been also associated with transcriptional activation triggered by light. In early photomorphogenic stages, light induces a quick redistribution of H2Bub marks in the Arabidopsis genome. Indeed, H2Bub is significantly overrepresented in coding regions of genes

upregulated by light, especially those that play important regulatory roles in photomorphogenesis (Bourbousse *et al*., 2012).

Despite increasing evidence linking the UPS with distinct chromatin remodeling machineries controlling photomorphogenesis, how their activities are interwined remains largely unknown. In this study, we propose that association of the CRL4-CDDD E3 ligase with H2B deubiquitinase (DUBm) facilitates both targeted destabilization of HY5 and repression of H2B deubiquitination to supress gene expression under non-inductive dark conditions.

CRL4-CDDD and DUBm complexes associate *in planta.*

CRL4-CDDD complexes have been described as multifunctional hubs in which different signaling pathways converge; i.e. light and photoperiod signaling, circadian function, DNA damage sensing, and more recently ABA perception and signaling. How these signals are integrated to trigger coordinated plant adaptive responses is a matter of discussion. In an effort to characterize CRL4-CDDD functions, our group demonstrated that these complexes display E3 ubiquitin ligase activity, where the DDA1 subunit acts as a novel type of substrate adaptor recognizing specific target proteins for ubiquitination (Irigoyen *et al*., 2014). Among potential targets for DDA1 recognition, we found SGF11, a protein involved in H2Bub deubiquitination and gene expression control in other model organisms. Previously, it had been proposed that both DET1 and DDA1 might participate in chromatin dynamics, although the molecular details of such activities and their functional relevance were unknown. In this context, DDA1-SGF11 interaction represented a new connection between CRL4-CDDD complexes and the chromatin remodeling machinery that we aimed to explore.

Similar to a previous study, showing that DDA1 specifically recognizes the targets for CRL4-CDDD E3 ligases; e.g. PYR/PYL receptors, SGF11 interacted only with DDA1 and not with other components of the CRL4-CDDD complex. In the case of COP10 physical interaction with SGF11 was observed by means of yeast two hybrids assays. However, such interaction could not be confirmed in planta when BiFC techniques were undertaken. Although we cannot exclude the possibility that COP190 and SGF11 construct were missexpressed in those assays, these results support the notion that, within the CRL4-CDDD complex, DDA1 acts as the substrate adaptor.

By using TAP technology, we confirmed that, similar to its counterparts in other organisms, SGF11 is part of the DUBm complex. Studies in yeasts have shown that Sgf11 subunit acts as a

scaffold protein for the rest of the complex and mediates its association to chromatin by physically interacting with nucleosomes (Lee *et al*., 2009). More specifically, Sgf11 binds the H3/H4 groove within the nucleosomes, which is in agreement with our data using protein chips showing that SGF11 does not recognize specific nude DNA sequences but it rather requires a chromatin context (data not shown). Our TAP assays also showed that, in addition to SGF11, the Arabidopsis DUBm included homologs for known subunits Sus1/ENY2 and Ubp8/USP22 (in yeast and mammals, respectively). These corresponded to proteins encoded by loci At3g27100 and At5g10790, which had not been previously characterized. According to Arabidopsis databases and nomenclature, we termed them ENY2 and UBP22. These results were confirmed when protein complexes were purified when UBP22 was used as a bait. Interaction between SGF11 and both ENY2 and UBP22 were confirmed by yeast two hybrids assays. No direct binding of ENY2 and UBP22 was observed which supported the idea that SGF11 is required to bring the whole DUBm together.

In yeast and animal, the DUBm acts as a subcomplex of the SAGA machinery. Indeed, in these organisms the DUBm comprises an additional subunit, Sgf73/ATXN3, which anchors the DUBm to the SAGA (Lee *et al*., 2009). Both phylogenetic analyses and TAP experiments did not result in identification of any protein with close homology to Sgf73/ATXN3 in Arabidopsis. However, a distant relative for Sgf73 may exist in *Physcomitrella* (Lee *et al*., 2009). Thus, to date, there is no evidence supporting the association of DUBm to SAGA in Arabidopsis, so they might act independently. The facts that SAGA components could not be found in SGF11 and UBP22 TAP assays and that there is no homolog for Sgf73/ATXN3 in vascular plants support this notion. Indeed, it has been shown that knock out of Sgf73 in fission yeast does not cause lethality. Therefore, SGF73 may not be essential for all organisms and association to SAGA might not be a requisite for DUB function. However, in light to our results, we cannot rule out the possibility that known or yet-to-be identified proteins have taken over Sgf73/ATXN3 function as a link between SAGA and DUBm. Accordingly, in our TAP assays we found additional proteins associated with SGF11-containing complexes. This is the case of TONNEAU1A and 1B, which control microtubule and chromosomal rearrangements that precede cell division. A potential function for TON1A and 1B in chromatin remodelling has not yet been described although it has been reported that, besides cytoplasmic localization, they can be also found in nuclei (reference). However, TON1 proteins were not present in TAP samples where UBP22 was used as bait, suggesting that their association to the DUBm is transient or spurious. Whether this is

the case or, on the contrary, TON1 proteins are bona-fide constituents of the Arabidopsis DUBm should be addressed in future studies.

The Arabidopsis DUBm acts as an H2Bub deubiquitinase.

Prior to this study, the function of the Arabidopsis DUBm complex had not been characterized. Subcellular localization assays using fusions to GFP for all identified subunits showed they localized exclusively in nuclei. This result is in agreement with their proposed function as regulators of histone modification at specific loci to regulate gene expression. According to this notion, Arabidopsis DUBm subunits were present in euchromatic regions within the nucleus and were almost excluded from heterochromatin, except in the case of ENY2.

Functional characterization of DUBm subunits could be accomplished in the case of SGF11 and UBP22 for which null mutants were available. As expected for a role of DUBm in H2Bub deubiquitination, deletion of UBP22 or SGF11 resulted in an increase in the overall state of H2Bub. On the contrary, overexpression of UBP22 but not that one of SGF11 promoted H2Bub deubiquitination, suggesting that, within the DUBm complex, UBP22 holds the deubiquitinase activity and very likely is the limiting factor of the complex for cleaving the ubiquitin peptide from H2Bub. These results were further supported by complementation of H2Bub deubiquitination defects in a yeast *Δubp8* mutant when transformed with an Arabidopsis UBP22 construct.

Our results using by TAP-purification, showed that ENY2 is part of a biochemically stable DUB module in Arabidopsis. However, the lack of mutants for ENY2 hampered its genetic and functional characterization in this study. In yeast and animals, Sus1/ENY2 is an evolutionary conserved component of both the H2Bm of the SAGA transcriptional co-activator. As well, in yeast, it is part of TREX-2 (Sus1-Sac3-Thp1-Cdc31), an mRNA export complex (Christoph *et al*., 2009). From our studies, it remains unclear whether ENY2 plays a direct role in H2B deubiquitination, or whether it modulates other transcriptional machineries. In this context, the fact that ENY2 fusions to GFP localized in both hetero– and euchromatin regions, different to UBP22 and SGF11 that colocalized almost exclusively with euchromatin, suggests that ENY2 can also act independently of the DUBm complex to control additional chromatin-associated processes (i.e. gene-silencing) in Arabidopsis. This potential independent function of ENY2 should be address in future studies.

Targeted destabilization of DUBm by CRL4-CDDD influences H2Bub levels.

CRL4-CDDD association with DUBm components pointed to a role of this E3 ligase in their targeted degradation as a means to control H2Bub deubiquitination under specific light conditions. In agreement with this notion, we found that SGF11, UBP22 and ENY2 are targeted for proteasomal degradation preferentially under dark conditions. Indeed, SGF11 polyubiquitination was increased in dark-grown seedlings. The fact that SGF11 was stabilized in *det1-1* mutants cultivated in the dark, indicated that the CRL4-CDDD complex mediates its degradation. This phenomenon was quite significant when plants were transferred from light to dark conditions whereas SGF11 levels remained stable over the time in plants exposed to continuous light. Our evidences are in agreement with a scenario in which CRL4-CDDD directly controls stability of SGF11, and likely that of UBP22 and ENY2, as we showed that these two proteins are also targeted for proteolysis by the UPS. According to this idea, *det1-1* mutants and oeDDA1-GFP plants displayed altered H2Bub levels that correlated with control of SGF11 accumulation and activity of DUBm. Thus, loss or gain of CRL4-CDDD function should lead to increased or reduced levels of SGF11, respectively, which should subsequently result in enhanced or reduced deubiquitination of H2Bub depending on the genetic backgrounds. We found that *sgf11-1 det1-1* double mutants accumulate more H2Bub than single *det1-1* mutants but further less than *sgf11-1* mutants under both conditions, indicating an additive effect of both mutations on H2Bub accumulation. However, these results could also be explained by a role of DET1 as a regulator of the activity of additional ubiquitin proteases acting on H2Bub. Noteworthy, no significant changes in H2Bub levels were observed in other CDDD mutants, such as *ddb1a* and *cop10-4* mutants, which could be explained by the fact that they represent very weak mutant alleles.

Recent studies have shown that the two RING E3 ligases HISTONE MONOUBIQUITINATION 1 and 2 (HUB1 and HUB2) together with E2 ubiquitin conjugation enzyme (AtUBC1/AtUBC2) promote H2B ubiquitination in Arabidopsis. This activity is required for activation of both floral repressor and photomorphogenic genes in Arabidopsis (Xu *et al*., 2009; Borbousse *et al*., 2012). It has been proposed that histone monoubiquitination activity by E3 ligase (HUB) may interfere with H2B deubiquitination by DUBm. In agreement with this idea, it has been recently observed that yeast Sgf11 competes with Bre1 (the yeast homolog for Arabidopsis HUB1/2) for binding the acidic patch at the nucleosome core particle (Gallego *et al*., 2016). In this context,

it would be plausible that CRL4-CDDD directly controls H2Bub abundance by controlling the stability or/and activity of HUB1/2 or AtUBC1/2 proteins. However, we could not observe *in vivo* association of CRL4-CDDD with these proteins, which does not support this model.

All together, our results imply a regulatory role of CRL4-CDDD in the H2B ubiquitination status by mediating the degradation of DUBm components in Arabidopsis. How such alteration affects the landscape of H2Bub at a genome wide scale needs yet to be determined. However, the fact that it affects global H2Bub levels points to a major role of CRL4-CDDD in controlling extensive abundance of this epigenetic mark throughout the chromatin. Moreover, defects in H2Bub in plants with severely altered CDDD function correlated with altered abundance of other epigenetic marks such as H3K36me3 and H3K4me3, which are associated to increased transcriptional activity. Current studies in our group aim to characterize how these epigenetic marks are redistributed at a genome scale depending on CRL4-CDDD and DUBm function, as well as the effect of the imposed light conditions, and how they are translated in specific changes in gene expression.

DUBm facilitates CRL4-CDDD mediated repression of photomorphogenesis.

Destabilization of SGF11 mediated by DET1 suggested that the DUBm complex and the E3 ubiquitin ligase CRL4-CDDD play antagonistic roles in controlling H2Bub-dependent gene expression. However, contrary to simple expectations, Arabidopsis double mutants combining the *det1-1* mutation with either loss of *SGF11* or *UBP22* function displayed an aggravated detiolated phenotype when grown in the dark. Under these conditions, *sgf11* and *ubp22* single mutants did not differ from wild type controls indicating a synergistic effect on *det1* mutation. These results suggested that both CRL4-CDDD and DUBm complexes collaborate to repress photomorphogenesis when the lights are off.

CRL4-CDDD complexes are known to facilitate degradation of transcription factor HY5 as a means to repress light development. Indeed, *det1* and *cop10* mutants display increased HY5 levels in the dark, which, in part, underlie their constitutively photomorphogenic phenotype. According to the notion that the DUBm complex facilitates repression of photomorphogenesis in close collaboration with CRL4-CDDD E3 ligases, increased HY5 abundance was observed in *det1-1 sgf11* double mutants. Very importantly, overexpression of *SGF11* was able to partially suppress the *det1-1* phenotype by reducing HY5 accumulation. This effect occurred

posttranscriptionally, as *HY5* mRNA levels were unaffected in those lines. Due to the fact that plants carrying the *det1-1* mutation express only 1% wild-type *DET1* transcript, we propose that increased SGF11 function makes remnant DET1 protein levels more efficient; i.e. to promote HY5 degradation.

An attractive scenario to explain such an increase in DET1 efficiency is that, by physically interaction, SGF11 helps to recruit DET1, together with the rest of the CRL4-CDDD complex, to light-responsive promoters were transcription factor HY5 is bound. This mechanism would foster HY5 destabilization under appropriate conditions; i.e. upon transition to dark conditions.

In view of the results of this study and those of other groups, we propose a model in which, by controlling HY5 and DUBm accumulation, CRL4-CDDD E3 ligases coordinately control H2Bub abundance at the promoter of light responsive genes levels and destabilization of HY5 to repress light-associated gene expression. In this process, the DUBm complex may facilitate recruitment of CRL4-CDDD to loci where both HY5 and H2Bub/deubiquitination machinery are acting. In parallel, histone deacetylation may increase the affinity of DET1 for non-acetylated H2B tails, further strengthening CRL4-CDDD association to chromatin to repress gene expression. Such a model would be in agreement with a recent study showing that DET1 can act as a transcriptional repressor (e.g. of *TOC1,* a master gene of the Arabidopsis circadian clock; Lau *et al*., 2012).

The fact that SGF11 associates with the COP9 signalosome (CSN) may represent and additional mechanism by which the DUBm modulates the activity of CRL4-CDDD in targeting specific proteins (i.e. HY5). Thus, it is possible that DUBm complexes recruit the CSN to the chromatin to facilitate, the deneddylation of CUL4, and therefore the assembly and disassembly of the CRL4-CDDD complex with is required for target recognition and ubiquitination.

Finally, it is plausible that the DUBm effect on CRL4-CDDD function is not limited to HY5 accumulation and may affect other targets as it has been shown that the defects in HY5 accumulation are not enough to explain all the phenotypes of *cop/det/fus* mutants. So, we can speculate that DUBm affects the stability of other targets of CRL4-CDDD (i.e. phyA, LAF1, HFR1, PIFs) or may modulate other CRL4-CDDD associated functions such transcriptional repression of specific genes (i.e. TOC1), being involved therefore in numerous biological processes controlled by these E3 ubiquitin ligases (circadian clock, DNA damage repair, flowering

control,…). Further studies will be required to fully demonstrate this hypothesis.

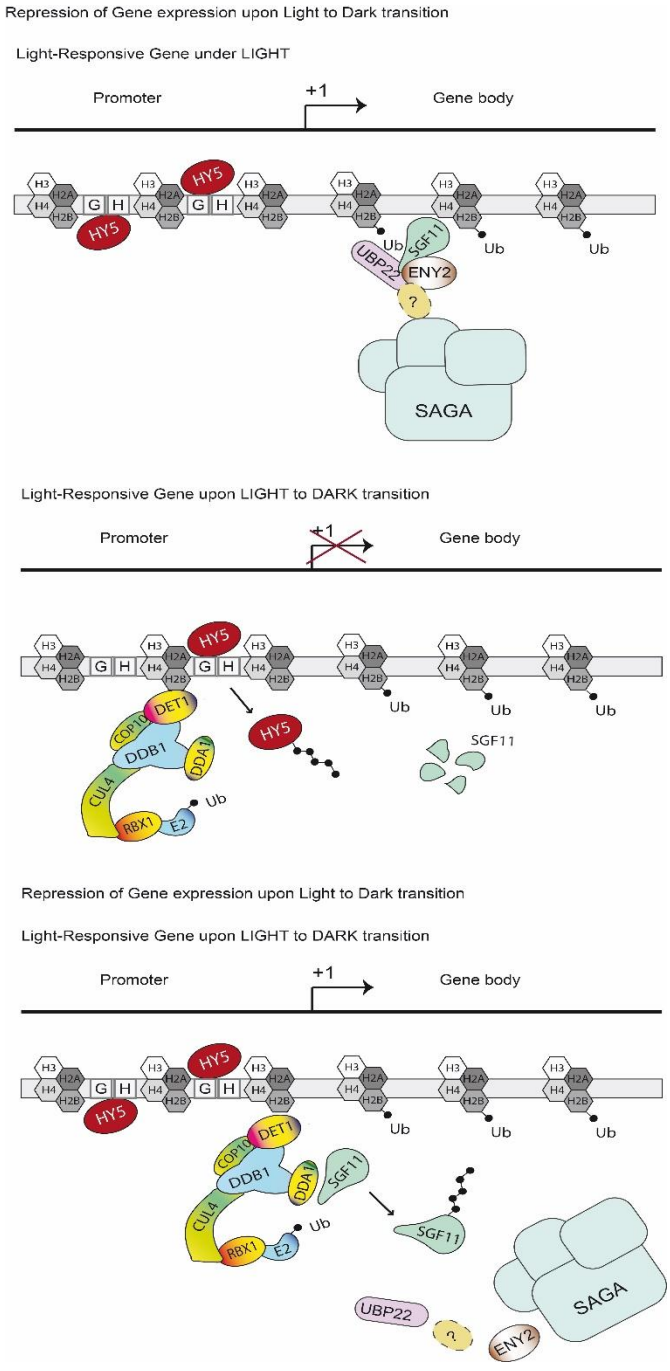


Figure 30. Model proposed for the coordinated action of CRL4-CDDD E3 ligases and DUBm complexes to control light-respnsive gene expression.

Interaction of DDA1, a substrate adaptor for CRL4-CDDD E3 ubiquitin ligases, with SGF11, a DUBm subunit, allows CRL4-CDDD/DUBm association. In this way, SGF11 helps to recruit CRL4-CDDD complexes to chromatin regions where HY5 is bound. Subsequently, CRL4-CDDD triggers

degradation of both HY5 and SGF11, limiting their activities as regulators of gene expression in response to light. Coupling of both activities may allow efficient modulation of the expression of light signaling genes (controlled by HY5) depending on the changing light conditions. This mechanism might be extensive to other biological processes regulated by CRL4-CDDD E3 ligases (i.e. circadian clock function, flowering transition, ABA signaling)

CONCLUSIONS

Conclusions.

1. UBP22, SGF11 and ENY2 are components of the DUBm complex in *Arabidopsis*, which mediates H2Bub deubiquitination

2. UBP22, SGF11, DET1 and DDA1 are localized in the nucleus, associated to transcriptionally-active *euchromatin, whereas ENY2 is also present in heterochromatic regions.*

3. SGF11 is a target of CRL4-CDDD ubiquitination and degradation by the 26S proteasome.

4. The abundance of the DUBm is controlled by the light conditions since SGF11, UBP22 and ENY2 are destabilized by the 26S proteasome preferentially in darkness.

5. Altered function of DET1 and DDA1 affects global H2Bub abundance and that of H3K36me3 and H3K4me3 histone marks, indicating a role of CRL4-CDDD complexes in the regulation of the epigenetic machinery. CRL4-CDDD effect on chromatin modification is likely associated to its function towards SGF11.

6. Loss of SGF11 or UBP22 function has a synergistic effect on the photomorphogenic defects of *det1* mutants, which correlates with their HY5 abundance, suggesting that the DUBm facilitates CRL4-CDDD-mediated repression of light signaling.

7. Overexpression of SGF11 ameliorates the phenotypic defects of *det1* mutants, suggesting that the CRL4-CDDD requires SGF11 for recruitment to the chromatin, recognition of HY5 and repression of light-responsive gene expression.

REFERENCES

References.

Azimzadeh J., Nacry P., Christodoulidou A., Drevensek S., Camilleri C., Amiour N., Parcy F., Pastuglia M., Bouchez D. (2008). *Arabidopsis* TONNEAU1 proteins are essential for preprophase band formation and interact with centrin. Plant Cell 20: 2146–2159.

Barneche F, Malapeira J, Mas P. (2014). The impact of chromatin dynamics on plant light responses and circadian clock function. J Exp Bot. 2014;65:2895–2913.

Bechtold, N., Ellis, J., and Pelletier, G. (1993). In planta Agrobacterium mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. C. R. Acad. Sci. Paris Life Sciences. 316, 15-18.

Belotserkovskaya, S. Oh, V.A. Bondarenko, G. Orphanides, V.M. Studitsky, D. Reinberg. (2003). FACT facilitates transcription-dependent nucleosome alteration Science, 301, pp. 1090–1093.

Benhamed M, Bertrand C, Servet C, Zhou DX. (2006). *Arabidopsis* GCN5, HD1, and TAF1/HAF2 interact to regulate histone acetylation required for light-responsive gene expression. Plant Cell., 18 (2006), pp. 2893–2903.

Benhamed M, Martin-Magniette ML, Taconnat L *et al*. 2008.Genome-scale Arabidopsis promoter array identifies targets of the histone acetyltransferase GCN5. The Plant Journal 56, 493–504.

Benvenuto, G., Formiggini, F., Laflamme, P., Malakhov, M., Bowler, C.(2002). The photomorphogenesis regulator DET1 binds the amino-terminal tail of histone H2B in a nucleosome context. Current Biology 12:1529-1534.

Berger SL. 2007. The complex language of chromatin regulation during transcription. Nature 447, 407–412.

Bergmüller E, Gehrig PM, Gruissem W. (2007) Characterization of post-translational modifications of histone H2B-variants isolated from *Arabidopsis thaliana.* J Proteome Res 6: 3655–3668.

Bernhardt A., Mooney S., Hellmann H. (2010). Arabidopsis DDB1a and DDB1b are critical for embryo development. Planta **232**: 555–566.

Bertrand C, Benhamed M, Li YF, Ayadi M, Lemonnier G, Renou JP, Delarue M, Zhou DX. (2005). Arabidopsis HAF2 gene encoding TATA-binding protein (TBP)-associated factor TAF1, is required to integrate light signals to regulate gene expression and growth. *Journal of Biological Chemistry* 280, 1465–1473.

Biedermann S. and Hellmann H. (2011). WD40 and CUL4-based E3 ligases: lubricating all aspects of life. Trends Plant Sci, 16 pp. 38–46.

Bohm L., Crane-Robinson, C., and Sautiere, P. 1980. Proteolytic digestion studies of chromatin core-histone structure. Identification of a limit peptide of histone H2A. Eur. J. Biochem. 106: 525-530.

Bourbousse C, Ahmed I, Roudier F, Zabulon G, Blondet E, Balzergue S, Colot V, Bowler C, Barneche F. (2012). Histone H2B monoubiquitination facilitates the rapid modulation of gene expression during Arabidopsis photomorphogenesis. PLoS Genetics 8, e1002825

Cao Y Dai Y Cui S Ma L. (2008). Histone H2B monoubiquitination in the chromatin of FLOWERING LOCUS C regulates flowering time in Arabidopsis. The Plant Cell 20, 2586–2602.

Casal JJ. (2013) Photoreceptor signaling networks in plant responses to shade. Annu Rev Plant Biol 64: 403–427.

Casal, J.J., Candia, A.N., Sellaro, R. (2014). Light perception and signalling by phytochrome A. Journal of Experimental Botany 65:2835-2845.

Casati P, Campi M, Chu F, Suzuki N, Maltby D, Guan S, Burlingame AL, Walbot V. 2008. Histone acetylation and chromatin remodeling are required for UV-B-dependent transcriptional activation of regulated genes in maize. The Plant Cell 20, 827–842.

Castells E., Molinier J., Drevensek S., Genschik P., Barneche F., Bowler C.(2010). det1-1-induced UV-C hyposensitivity through UVR3 and PHR1 photolyase gene over expression. Plant J. 63: 392–404.

Chamovitz, D.A. (2009). Revisiting the COP9 signalosome as a transcriptional regulator. EMBO Reports 10:352-358.

Charron JB, He H, Elling AA, Deng XW (2009) Dynamic landscapes of four histone modifications during deetiolation in *Arabidopsis*. Plant Cell 21: 3732–3748.

Chassy, B.M., and Flickinger, J.L**.** (1987). Transformation of Lactobacillus casei by electroporation. FEMS Microbiology Letters. 44**,** 173-177.

Chaves I., Pokorny R., Byrdin M., Hoang N., Ritz T., Brettel K., Essen L.O., van der Horst G.T., Batschauer A., Ahmad M. (2011). The cryptochromes: blue light photoreceptors in plants and animals. Annu. Rev. Plant Biol.;62:335–364.

Chen Z., Tian L. (2007). Roles of dynamic and reversible histone acetylation in plant development and polyploidy. Biochim. Biophys. Acta 1769, 295–307.

Chen, H., Huang, X., Gusmaroli, G., Terzaghi, W., Lau, O.S., Yanagawa, Y., Zhang, Y., Li, J., Lee, J.H., Zhu, D., Deng, X.W. (2010). Arabidopsis CULLIN4-damaged DNA binding protein 1 interacts with CONSTITUTIVELY PHOTOMORPHOGENIC1-SUPPRESSOR OF PHYA complexes to regulate photomorphogenesis and flowering time. Plant Cell 22:108-123.

Chen, H., Shen, Y., Tang, X., Yu, L., Wang, J., Guo, L., Zhang, Y., Zhang, H., Feng, S., Strickland, E., Zheng, N., Deng, X.W. (2006). Arabidopsis CULLIN4 forms an E3 ubiquitin ligase with RBX1 and the CDD complex in mediating light control of development. Plant Cell 18:1991-2004.

Chory, J., Peto, C., Feinbaum, R., Pratt, L., Ausubel, F. (1989). Arabidopsis thaliana mutant that develops as a light-grown plant in the absence of light. Cell 58:991-999.

Christoph K, Maren S, Sheila L, Divyang J, Dieter K, Murray S, Ed H, and Alwin K. (2009). Mutational Uncoupling of the Role of Sus1 in Nuclear Pore Complex Targeting of an mRNA Export Complex and Histone H2B Deubiquitination. J Biol Chem. 284(18): 12049–12056.

Chua YL Brown AP Gray JC. 2001. Targeted histone acetylation and altered nuclease accessibility over short regions of the pea plastocyanin gene. The Plant Cell 13, 599–612.

Chua YL Watson LA Gray JC. 2003. The transcriptional enhancer of the pea plastocyanin gene associates with the nuclear matrix and regulates gene expression through histone acetylation. The Plant Cell 15, 1468–1479.

Ciolfi A, Sessa G, Sassi M, Possenti M, Salvucci S, Carabelli M, Morelli G, Ruberti I. 2013. Dynamics of the shade-avoidance response in Arabidopsis. Plant Physiology 163, 331–353.

Crocco, C.D., Holm, M., Yanovsky, M.J., and Botto, J.F. (2010). AtBBX21 and COP1 genetically interact in the regulation of shade avoidance. Plant J. 64: 551–562.

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR. 2010. Abscisic acid: Emergence of a core signaling network. Annu Rev Plant Biol **61**: 651–679.

Daniel J.A., Grant P.A. (2007). Multi-tasking on chromatin with the SAGA coactivator complexes. Mutat. Res. ;618:135–148.

Daniel, J., Torok, M., Sun, Z., Schieltz, D., Allis, C., Yates, J. III, *etal*.(2004). Deubiquitination ofhistone H2B by a yeast acetyltransferase complex regulates transcription. *J.Biol.Chem.* 279,1867–1871.

Davière, J., and Achard, P. (2016). A pivotal role of DELLAs in regulating multiple hormone signals. Molecular Plant 9:10-20.

Deblaere, R. *et al*. (1985). Efficient octopine Ti plasmid-derived vectors for Agrobacterium-mediated gene transfer to plants. Nucleic acids research 13, 4777-4788.

Deshaies, R. J. and Joazeiro, C. A. (2009). RING domain E3 ubiquitin ligases. Annu. Rev. Biochem. 78, 399-434.

Devlin PF, Yanovsky MJ, Kay SA. 2003. A genomic analysis of the shade avoidance response in Arabidopsis. Plant Physiology 133, 1617–1629.

Dong, J., Tang, D., Gao, Z., Yu, R., Li, K., He, H., Terzaghi, W., Deng, X.W., Chen, H. (2014). Arabidopsis DE-ETIOLATED1 represses photomorphogenesis by positively regulating phytochrome-interacting factors in the dark. Plant Cell 26:3630-3645.

Dong, J., Terzaghi, W., Deng, X.W., Chen, H. (2015). Multiple photomorphogenic repressors work in concert to regulate Arabidopsis seedling development. Plant Signalling and Behaviour 10:e1011934.

Doyle, J.J., and Doyle, J.L. (1990). Isolation of plant DNA from fresh tissue. Focus. 12, 13-15.

Earley K., Shook M., Brower-Toland B., Hicks L., Pikaard C. (2007). *In vitro* specificities of Arabidopsis co-activator histone acetyltransferases: implications for histone hyperacetylation in gene activation. Plant J. 52, 615–626.

Endo, M., S. Nakamura, *et al*. (2005). "Phytochrome B in the mesophyll delays flowering by suppressing FLOWERING LOCUS T expression in Arabidopsis vascular bundles." Plant Cell 17(7): 1941-1952.

Favory JJ, Stec A, Gruber H, Rizzini L, Oravecz A, Funk M, Albert A, Cloix C, Jenkins GI, Oakeley EJ, Seidlitz HK, Nagy F, Ulm R. (2009). Interaction of COP1 and UVR8 regulates UV-B-induced photomorphogenesis and stress acclimation in Arabidopsis. EMBO J. 2009 Mar 4; 28(5):591-601.

Feng J, Shen WH. (2014) Dynamic regulation and function of histone monoubiquitination in plants. Front Plant Sci 5: 83.

Fernando, V.C.D., Schroeder D.F. (2015). Genetic interactions between DET1 and intermediate genes in Arabidopsis ABA signalling. Plant Science 239:166-179.

Fierz B, Chatterjee C, McGinty RK, Bar-Dagan M, Raleigh DP, Muir TW 2011. Histone H2B ubiquitylation disrupts local and higher-order chromatin compaction. Nat Chem Biol 7: 113–119.

Fleming AB, Kao CF, Hillyer C, Pikaart M, Osley MA. (2008). H2B ubiquitylation plays a role in nucleosome dynamics during transcription elongation. Mol Cell.;31:57–66.

Fleury, D., Himanen, K., Cnops, G. et al. (2007) The Arabidopsis thaliana homolog of yeast BRE1 has a function in cell cycle regulation during early leaf and root growth. Plant Cell, 19,417–432.

Fornara F, Panigrahi KCS, Gissot L, Sauerbrunn N, Rühl M, Jarillo JA, Coupland G. (2009). Arabidopsis DOF transcription factors act redundantly to reduce CONSTANS expression and are essential for a photoperiodic flowering response. Dev Cell 17: 75–86.

Gallego L D., Steger M G, Polyansky A A., Schubert T, Zagrovic B, Zheng N, Clausen T, Herzog F, and Köhlera A. (2016). Structural mechanism for the recognition and ubiquitination of a single nucleosome residue by Rad6–Bre1. Proc Natl Acad Sci U S A. 113(38): 10553–10558.

Ganpudi, A.L, and Schroeder, D.F. (2013). Genetic interactions of Arabidopsis thaliana damaged DNA binding protein 1B (DDB1B) with DDB1A, DET1, and COP1. Genes Genomes Genetics (G3) (Bethesda) 3:493-503.

Govind C.K, Zhang F, Qiu H, Hofmeyer K, Hinnebusch A.G. (2007). Gcn5 promotes acetylation, eviction, and methylation of nucleosomes in transcribed coding regions. Mol Cell 25: 31–42.

Grant, P., Duggan, L., Cote, J., Roberts, S. M., Brownell, J. E., Candau, R., *etal*. (1997).Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleoso malhistones: characterizationof an Ada complex and the SAGA (Spt/Ada) complex. *GenesDev.* 11,1640–1650.

Gu X Jiang D Wang Y Bachmair A He Y. 2009. Repression of the floral transition via histone H2B monoubiquitination. The Plant Journal 57, 522–533.

Guo L Zhou J Elling AA Charron JB Deng XW. 2008. Histone modifications and expression of light-regulated genes in Arabidopsis are cooperatively influenced by changing light conditions. Plant Physiology 147, 2070–2083.

Guo L., Nezames C.D., Sheng L., Deng X., Wei N. (2013). Cullin-RING ubiquitin ligase family in plant abiotic stress pathways. J. Integr. Plant Biol. 55: 21–30.

Gurskiy D., Orlova A., Vorobyeva N., Nabirochkina E., Krasnov A,, Shidlovskii Y., Georgieva S., and Daria Kopytova. (2012). The DUBm subunit Sgf11 is required for mRNA export and interacts with Cbp80 in Drosophila. Nucleic Acids Res. Nov; 40(21): 10689–10700.

Halliday K J.,. Martínez-García J. F., and Josse E. (2009). Integration of Light and Auxin Signaling. Cold Spring Harb Perspect Biol. 2009 Dec; 1(6): a001586.

Hanahan, D. (1985). Techniques for transformation of E.coli. In: DNA cloning. (ed.Glover, D.M.) Oxford, Washington DC. IRL Press Limited. **1,** 109-136.

Helmlinger, S. Hardy, S. Sasorith, F. Klein, F. Robert, C. Weber, L. Miguet, N. Potier, A. Van-Dorsselaer, J.M. Wurtz, *et al.* (2004). Ataxin-7 is a subunit of GCN5 histone acetyltransferase-containing complexes. Hum. Mol. Genet., 13, pp. 1257–1265

Henry K. W., Wyce A, Lo W. S., Duggan L. J., Emre N.C.T., Kao CF., Pillus L., Shilatifard A., Osley MA., Berger SL. (2003).Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. Genes Dev. ;17:2648–2663.

Hershko A & Ciechanover A. 1998; The ubiquitin system. *Annu. Rev. Biochem.* **67**: 425–479.

Hicke L. (2001). Protein regulation by monoubiquitin. Nat Rev 2:195–201.

Himanen K, Woloszynska M, Boccardi TM, De Groeve S, Nelissen H, Bruno L, Vuylsteke M, Van Lijsebettens M (2012). Histone H2B monoubiquitination is required to reach maximal transcript levels of circadian clock genes in Arabidopsis. Plant J 72: 249–260.

Himanen K., Woloszynska M., Boccardi TM., De Groeve S., Nelissen H., Bruno L., Vuylsteke M., Van Lijsebettens M. (2012). Histone H2B monoubiquitination is required to reach maximal transcript levels of circadian clock genes in Arabidopsis. The Plant Journal 72, 249–260.

Hochstrasser M. 1996. Protein degradation or regulation: Ub the judge. Cell 84, 813–815.

Hoecker U, Tepperman JM, Quail PH. (1999). SPA1, a WD-repeat protein specific to phytochrome A signal transduction. Science 284: 496-499.

Holm M, Ma LG, Qu LJ, Deng XW. (2002) Two interacting bZIP proteins are direct targets of COP1-mediated control of light-dependent gene expression in Arabidopsis. Genes Dev 16: 1247–1259.

Holsters, M., de Waele, D., Depicker, A., Messens, E., van Montagu, M., and Schell, J. (1978). Transfection and transformation of Agrobacterium tumefaciens. Mol Gen Genet. 163, 181-187.

Hornitschek P, Kohnen MV, Lorrain S *et al*. 2012. Phytochrome interacting factors 4 and 5 control seedling growth in changing light conditions by directly controlling auxin signaling. The Plant Journal 71, 699–711.

Huang, X., Ouyang, X., Deng, X.W. (2014). Beyond repression of photomorphogenesis: role switching of COP/DET/FUS in light signalling. Current Opinion in Plant Biology 21:96-103.

Huisinga K.L, Pugh B.F. (2004). A genome-wide housekeeping role for TFIID and a highly regulated stress-related role for SAGA in *Saccharomyces cerevisiae*. Mol Cell 13: 573–585.

Ikeda, F. and Dikic, I. (2008). Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' Review Series. EMBO Rep., 9 , pp. 536–542.

Imaizumi, T. and Kay, S.A. (2006). Photoperiodic control of flowering: not only by coincidence. Trends Plant Sci 11: 550–558.

Ingvarsdottir K, Krogan NJ, Emre NC, Wyce A, Thompson NJ, Emili A, Hughes TR, Greenblatt JF, Berger SL (2005) H2B ubiquitin protease Ubp8 and Sgf11 constitute a discrete

functional module within the *Saccharomyces cerevisiae* SAGA complex. Mol Cell Biol 25: 1162–1172 .

Irigoyen, M. L., Iniesto, E., Rodriguez, L., Puga, M. I., Yanagawa, Y., Pick, E., *et al*. (2014). Targeted degradation of abscisic acid receptors is mediated by the ubiquitin ligase substrate adaptor DDA1 in Arabidopsis. Plant Cell 26, 712–728.

Jackson S. and Xiong Y. (2009). CRL4s: the CUL4-RING E3 ubiquitin ligases. *Trends Biochem. Sci.* 34, 562-570.

Jang IC, Chung PJ, Hemmes H, Jung C, Chua NH. (2011). Rapid and reversible light-mediated chromatin modifications of Arabidopsis phytochrome A locus. *The Plant Cell* 23, 459–470.

Jang S., Marchal V., Panigrahi K.C., Wenkel S., Soppe W., Deng X.W., Valverde F., Coupland G. (2008). Arabidopsis COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. EMBO J. 27: 1277–1288.

Jiao Y, Lau OS, Deng XW (2007). Light-regulated transcriptional networks in higher plants. Nat Rev Genet 8 217–230.

Johnson L., Mollah S., Garcia BA., Muratore TL., Shabanowitz J., Hunt DF., and Jacobsen SE. (2004). Mass spectrometry analysis of Arabidopsis histone H3 reveals distinct combinations of post-translational modifications. Nucleic Acids Research 32, 6511–6518.

Kang, M., Yoo, S., Kwon, H., Lee, B., Cho, J., Noh, Y., Paek, N. (2015). Negative regulatory roles of DE-ETIOLATED1 in flowering time in Arabidopsis. Scientific Reports 5: 9728.

Katan-Khaykovich, Y., and K. Struhl. (2002). Dynamics of global histone acetylation and deacetylation in vivo: rapid restoration of normal histone acetylation status upon removal of activators and repressors. Genes Dev. 16:743–752.

Kendrick RE, Kronenberg GHM (1994) Photomorphogenesis in Plants. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Koehler C, Bonnet J, Stierle M, Romier C, Devys D, Kieffer B.(2014) DNA binding by Sgf11 protein affects histone H2B deubiquitination by Spt-Ada-Gcn5-acetyltransferase (SAGA).J Biol Chem. 2014 Mar 28;289(13):8989-99.

Koncz, C. S., J. The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. Molecular and General Genetics 204, 383-396 (1986).

Kong, S., Okajima, K. (2016). Diverse photoreceptors and light responses in plants. Journal of Plant Research 129:111-114.

Koutelou E, Hirsch C, Dent S. (2010). Multiple faces of the SAGA complex. Curr Opin Cell Biol 22: 374–382.

Krasnov A.N, Kurshakova M.M, Ramensky V.E, Mardanov P.V, Nabirochkina E.N, Georgieva S.G (2005). A retrocopy of a gene can functionally displace the source gene in evolution. Nucleic Acids Res. 33:6654–6661.

Kwok, S. F. *et al.* Arabidopsis homologs of a c-Jun coactivator are present both in monomeric form and in the COP9 complex, and their abundance is differentially affected by the pleiotropic cop/det/fus mutations. *The Plant cell* **10**, 1779-1790 (1998).

Lang G, Bonnet J, Umlauf D, Karmodiya K, Koffler J, Stierle M, Devys D, Tora L. (2011). The tightly controlled deubiquitination activity of the human SAGA complex differentially modifies distinct gene regulatory elements. Mol Cell Biol. 31:3734–3744.

Lau OS, Deng XW. (2009). Effect of Arabidopsis COP10 ubiquitin E2 enhancement activity across E2 families and functional conservation among its canonical homologues. Biochem J. 418:683–690.

Lau, O.S., and Deng, X.W. (2012). The photomorphogenic repressors COP1 and DET1: 20 years later. Trends in Plant Science 17:584-593.

Lau, O.S., Huang, X., Charron, J., Lee, J., Li, G., Deng, X.W. (2011). Interaction of Arabidopsis DET1 with CCA1 and LHY in mediating transcriptional repression in the plant circadian clock. Molecular Cell 43:703-712.

Lee K., Swanson S., Florens L., Washburn M., Workman J. (2009). Yeast Sgf73/Ataxin-7 serves to anchor the deubiquitination module into both SAGA and Slik (SALSA) HAT complexes. Epigenetics Chromatin 2:2.

Lee KK, and Workman JL (2007) Histone acetyltransferase complexes: one size doesn't fit all. Nat Rev 8: 284–295.

Leivar P, Monte E, Cohn MM, Quail PH (2012) Phytochrome signaling in green Arabidopsis seedlings: impact assessment of a mutually negative phyB-PIF feedback loop. Mol Plant 5:734–749.

Leivar, P., Monte, E. (2014). PIFs: systems integrators in plant development. Plant Cell 26:56-78.

Leivar, P., Monte, E., Oka, Y., Liu, T., Carle, C., Castillon, A., Huq, E., Quai, l P.H (2008). Multiple phytochrome-interacting bHLH transcription factors repress premature seedling photomorphogenesis in darkness. Current Biology 18:1815-1823.

Li, J., Li, G., Wang, H., and Wang Deng, X. (2011). Phytochrome signaling mechanisms. The Arabidopsis Book 9: e0148, doi/10.1199/tab.0148

Li, K., Gao, Z., He, H., Terzaghi, W., Fan, L., Deng, X.W, Chen, H. (2015). Arabidopsis DET1 represses photomorphogenesis in part by negatively regulating DELLA protein abundance in darkness. Molecular Plant 8:622-630.

Lian, H.L., He, S.B., Zhang, Y.C., Zhu, D.M., Zhang, J.Y., Jia, K.P., Sun, S.X., Li, L.,Yang, H.Q. (20 11). Blue-light-dependent interaction of cryptochrome 1 with SPA1 defines a dynamic signaling mechanism. Genes Dev. **25**: 1023–1028.

Liu J., Furukawa M., Matsumoto T., Xiong Y. (2002). NEDD8 modification of CUL1 dissociates p120(CAND1), an inhibitor of CUL1-SKP1 binding and SCF ligases. Mol. Cell 10, 1511–1518.

Liu X, Chen CY, Wang KC, *et al*. (2013). PHYTOCHROME INTERACTING FACTOR3 associates with the histone deacetylase HDA15 in repression of chlorophyll biosynthesis and photosynthesis in etiolated Arabidopsis seedlings. *The Plant Cell* 25, 1258–1273.

Liu Y, Koornneef M, Soppe WJ. (2007) The absence of histone H2B monoubiquitination in the Arabidopsis hub1 (rdo4) mutant reveals a role for chromatin remodeling in seed dormancy. Plant Cell 19: 433–444.

Liu, B., Yang, Z., Gomez, A., Liu, B., Lin, C., Oka, Y. (2016). Signalling mechanisms of plant cryptochromes in Arabidopsis thaliana. Journal of Plant Research 129:137-148.

Liu, L., Y. Zhang, et al. (2010). "An efficient system to detect protein ubiquitination by agroinfiltration in Nicotiana benthamiana." Plant J 61(5): 893-903.

Liu, L.-J., Zhang, Y.-C., Li, Q.-H., Sang, Y., Mao, J., Lian, H.-L., Wang, L., and Yang, H.Q. (2008). COP1-mediated ubiquitination of CONSTANS is implicated in cryptochrome regulation of flowering in Arabidopsis. Plant Cell 20: 292–306.

Lopez-Juez E, Dillon E, Magyar Z, Khan S, Hazeldine S, de Jager SM, Murray JA, Beemster GT, Bogre L, Shanahan H. 2008. Distinct light-initiated gene expression and cell cycle programs in the shoot apex and cotyledons of Arabidopsis. The Plant Cell 20, 947–968.

Lu, X., Zhou, C., Xu, P., Luo, Q., Lian, H., Yang, H. (2015). Red-light-dependent interaction of phyB with SPA1 promotes COP1–SPA1 dissociation and photomorphogenic development in Arabidopsis. Molecular Plant 8:467-478.

Ma L, Sun N, Liu X, Jiao Y, Zhao H, Deng XW. (2005). Organ-specific expression of Arabidopsis genome during development. Plant Physiology 138, 80–91.

Mao Y., Pavangadkar K. A., Thomashow M. F., Triezenberg S. J. (2006). Physical and functional interactions of Arabidopsis ADA2 transcriptional coactivator proteins with the acetyltransferase GCN5 and with the cold-induced transcription factor CBF1. Biochim. Biophys. Acta 1759, 69–79.

Maxwell, B.B., Andersson, C.R., Pool, D.S., Kay, S.A., Chory, J. (2003). HY5, Circadian Clock-Associated 1, and a cis-element, DET1 dark response element, mediate DET1 regulation of Chlorophyll a/b-Binding Protein 2 expression. Plant Physiology 133:1565-1577.

Menges, M. & Murray, J. A. Synchronous Arabidopsis suspension cultures for analysis of cell-cycle gene activity. The Plant journal : for cell and molecular biology 30, 203-212 (2002).

Minsky N, Shema E, Field Y, Schuster M, Segal E, Oren M (2008) Monoubiquitinated H2B is associated with the transcribed region of highly expressed genes in human cells. Nat Cell Biol 10: 483–488.

Mittler R, Blumwald E. 2010. Genetic engineering for modern agriculture: challenges and perspectives. Annual Review of Plant Biology 61: 443–462.

Moraga F. and Aquea F. (2015). Composition of the SAGA complex in plants and its role in controlling gene expression in response to abiotic stresses. Front Plant Sci. 6: 865.

Näär, A. M., Lemon, B. D., and Tjian, R. (2001).Transcriptional coactivatorcomplexes. *Annu.Rev.Biochem.* 70,475–501.doi: 10.1146/annurev.biochem.70.1.475.

Nakagawa, T. *et al.* (2007). Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *Journal of bioscience and bioengineering* 104, 34-41.

Nakashima K., Yamaguchi-Shinozaki K. (2013). ABA signaling in stress-response and seed development. Plant Cell Rep. 32 959–970.

Offermann S, Danker T, Dreymuller D, Kalamajka R, Topsch S, Weyand K, Peterhansel C. 2006. Illumination is necessary and sufficient to induce histone acetylation independent of transcriptional activity at the C4-specific phosphoenolpyruvate carboxylase promoter in maize. Plant Physiology 141, 1078–1088.

Olma M.H., Roy M., Le Bihan T., Sumara I., Maerki S., Larsen B., Quadroni M., Peter M., Tyers M., Pintard L. (2009). An interaction network of the mammalian COP9 signalosome identifies Dda1 as a core subunit of multiple Cul4-based E3 ligases. J. Cell Sci. 122: 1035–1044.

Oshikawa K, Matsumoto M, Yada M, Kamura T, Hatakeyama S, Nakayama KI. (2003). Preferential interaction of TIP120A with Cul1 that is not modified by NEDD8 and not associated with Skp1. Biochem Biophys Res Commun, 303:1209–1216.

Osterlund MT, Wei N, Deng XW. (2000b) The roles of photoreceptor systems and the COP1-targeted destabilization of HY5 in light control of Arabidopsis seedling development. Plant Physiol 124: 1520–1524.

Osterlund, M.T., Hardtke ,C,S., Wei, N., Deng, X.W. (2000a). Targeted destabilization of HY5 during light-regulated development of Arabidopsis. Nature 405:462-466.

Oyama, T., Shumura, Y., and Okada, K. (1997). The Arabidopsis HY5 gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyls. Genes Dev. 11: 2983–2994.

Pandey R., Muller A., Napoli C. A., Selinger D. A., Pikaard C. S., Richards E. J., et al. . (2002). Analysis of histone acetyltransferase and histone deacetylase families of *Arabidopsis thaliana* suggests functional diversification of chromatin modification among multicellular eukaryotes. Nucleic Acids Res. 30, 5036–5055.

Park, B.S., Eo, H.J., Jang, I, Kang, H., Song, J.T., Seo, H.S. (2010). Ubiquitination of LHY by SINAT5 regulates flowering time and is inhibited by DET1. Biochemical and Biophysical Research Communications 398:242-246.

Pavri, B. Zhu, G. Li, P. Trojer, S. Mandal, A. Shilatifard, D. Reinberg. (2006). Histone H2B monoubiquitination functions cooperatively with FACT to regulate elongation by RNA polymerase II. Cell, 125, pp. 703–717.

Pazhouhandeh, M., Molinier, J., Berr, A., Genschik, P. (2011). MSI4/FVE interacts with CUL4-DDB1 and a PRC2-like complex to control epigenetic regulation of flowering time in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America 108:3430-3435.

Pepper A., Delaney T., Washburn T., Poole D. & Chory J. (1994). DET1, a negative regulator of light-mediated development and gene expression in Arabidopsis, encodes a novel nuclear localized protein. Cell 78, 109–116.

Pepper, A., Delaney, T., Washburn, T., Poole, D., Chory, J. (1994). DET1, a negative regulator of light-mediated development and gene expression in Arabidopsis, encodes a novel nuclear-localized protein. Cell 78:109-116.

Pepper, A.E., Chory, J. (1997). Extragenic suppressors of the Arabidopsis det1 mutant identify elements of flowering-time and lightresponse regulatory pathways. Genetics 145:1125- 1137.

Pick E, Lau OS, Tsuge T, Menon S, Tong Y, Dohmae N, Plafker SM, Deng XW, Wei N (2007) Mammalian DET1 regulates Cul4A activity and forms stable complexes with E2 ubiquitin-conjugating enzymes. Mol Cell Biol 27: 4708–4719.

Pray-Grant MG, Schieltz D, McMahon SJ, Wood JM, Kennedy EL, Cook RG, *et al*. 2002. novel SLIK histone acetyltransferase complex functions in the yeast retrograde response pathway. Mol Cell Biol. ;22:8774–8786.

Rizzini L., Favory J.J., Cloix C., Faggionato D., O'Hara A., Kaiserli E., Baumeister R., Schafer E., Nagy F., Jenkins G.I., Ulm R. Perception of UV-B by the Arabidopsis UVR8 protein. Science. 2011;332:103–106.

Rodriguez-Navarro S. (2009). Insights into SAGA function during gene expression. EMBO Rep 10: 843–850.

Rodríguez-Navarro, S., Fischer, T., Luo, M.J., Antúnez, O., Brettschneider, S., Lechner, J., Pérez-Ortín, J.E., Reed, R. E. (2004). Sus1, a Functional Component of the SAGA Histone Acetylase Complex and the Nuclear Pore-Associated mRNA Export Machinery. Hurt Cell, 116, pp. 75–86.

Ronchi A, Farina G, Gozzo F, Tonelli C (1997) Effects of a triazolic fungicide on maize plant metabolism: modifications of transcript abundance in resistance-related pathways. Plant Sci 130: 51-62.

Roudier F Ahmed I Berard Cet al. (2011). Integrative epigenomic mapping defines four main chromatin states in Arabidopsis. EMBO Journal 30, 1928–1938.

Rubio S., Rodrigues A., Saez A., Dizon M.B., Galle A., Kim T.H., Santiago J., Flexas J., Schroeder J.I., Rodriguez P.L. (2009). Triple loss of function of protein phosphatases type 2C leads to partial constitutive response to endogenous abscisic acid. Plant Physiol. 150: 1345–1355.

Saez A., Robert N., Maktabi M.H., Schroeder J.I., Serrano R., Rodriguez P.L. (2006). Enhancement of abscisic acid sensitivity and reduction of water consumption in Arabidopsis by combined inactivation of the protein phosphatases type 2C ABI1 and HAB1. Plant Physiol. 141: 1389–1399.

Saijo Y, Sullivan JA, Wang H, Yang J, Shen Y, Rubio V, Ma L, Hoecker U, Deng XW. (2003) The COP1-SPA1 interaction defines a critical step in phytochrome A-mediated regulation of HY5 activity. Genes Dev 17: 2642–2647.

Sambrook, J., Fritsch, E. F. & Maniatis, T. Molecular cloning: A Laboratory Manual. 2nd edition. . *Cold Spring Harbor Laboratory Press, N.Y.* (1989).

Schroeder, D.F., Gahrtz, M., Maxwell, B.B., Cook, R.K., Kan, J.M., Alonso, J.M., Ecker, J.R., Chory ,J. (2002). De-etiolated 1 and damaged DNA binding protein 1 interact to regulate Arabidopsis photomorphogenesis. Current Biology 12:1462-1472.

Seo, H.S., Watanabe, E., Tokutomi, S., Nagatani, A., and Chua, N.H. **(**2004). Photoreceptor ubiquitination by COP1 E3 ligase desensitizes phytochrome A signaling. Genes Dev. 18**,** 617–622.

Seo, H.S., Yang, J.Y., Ishikawa, M., Bolle, C., Ballesteros, M.L., Chua, N.H. (2003). LAF1 ubiquitination by COP1 controls photomorphogenesis and is stimulated by SPA1. Nature 423:995-999.

Servet C Conde e Silva N Zhou DX. 2010. Histone acetyltransferase AtGCN5/HAG1 is a versatile regulator of developmental and inducible gene expression in Arabidopsis. Molecular Plant 3, 670–677.

Shahbazian M. D., and Grunstein M. (2007). Functions of site-specific histone acetylation and deacetylation. Annu. Rev. Biochem. 76, 75–100.

Sheerin, D.J., Menon, C., zur Oven-Krockhaus, S., Enderle. B., Zhu, L., Johnen, P., Schleifenbaum, F., Stierhof, Y,D., Huq, E., Hiltbrunner, A. (2015). Light-activated phytochrome A and B interact with members of the SPA family to promote photomorphogenesis in Arabidopsis by reorganizing the COP1/SPA complex. Plant Cell 27:189-201.

Shi, H., Wang, X., Mo, X., Tang, C., Zhong, S., Deng, X.W. (2015). Arabidopsis DET1 degrades HFR1 but stabilizes PIF1 to precisely regulate seed germination. Proceedings of the National Academy of Sciences of the United States of America 112:3817-3822.

Shilatifard A. 2006. Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. Annual Review of Biochemistry 75, 243–269.

Smalle J., Vierstra RD. (2004). The ubiquitin 26S proteasome proteolytic pathway. Annual Review of Plant Biology 55, 555–590.

Somers DE, Fujiwara S, Kim WY, Suh SS (2007) Posttranslational photomodulation of circadian amplitude. Cold Spring Harb Symp Quant Biol 72: 193–200.

Song, H., Carré, I. (2005). DET1 regulates the proteasomal degradation of LHY, a component of the Arabidopsis circadian clock. Plant Molecular Biology 57:761-771.

Spedale G Timmers HT Pijnappel WW. 2012. ATAC-king the complexity of SAGA during evolution. Genes and Development 26, 527–541.

Srivastava R., Rai K. M., Pandey B., Singh S. P., Sawant S. V. (2015). Spt-Ada-Gcn5-Acetyltransferase (SAGA) complex in plants: genome wide identification, evolutionary conservation and functional determination. PLoS ONE 10:e0134709.

Sterner DE, Grant PA, Roberts SM, Duggan LJ, Belotserkovskaya R, Pacella LA, *et al*. (1999). Functional organization of the yeast SAGA complex: distinct components involved in structural integrity, nucleosome acetylation, and TATA-binding protein interaction. Mol Cell Biol. 19:86–98.

Stockinger E. J., Mao Y., Regier M. K., Triezenberg S. J., Thomashow M. F. (2001). Transcriptional adaptor and histone acetyltransferase proteins in Arabidopsis and their interactions with CBF1, a transcriptional activator involved in cold-regulated gene expression. Nucleic Acids Res. 29, 1524–1533.

Sullivan J.A. and Deng, X.W. 2003. From seed to seed: The role of photoreceptors in Arabidopsis development. Dev. Biol. 260: 289-297.

Sun ZW, and Allis CD. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature 418, 104–108.

Suzuki G., Yanagawa, Y., Kwok, S.F., Matsui, M., and Deng, X.W. 2002. *Arabidopsis* COP10 is an ubiquitin-conjugating enzyme variant that acts together with COP1 and the COP9 signalosome in repressing photomorphogenesis. Genes & Dev. 16**:** 554-559.

Swerdlow P.S., Schuster, T., and Finley, D. 1990. A conserved sequence in histone H2A which is a ubiquitination site in higher eucaryotes is not required for growth in Saccharomyces cerevisiae. Mol. Cell. Biol. 10: 4905-4911.

Tepperman, J.M., Zhu, T., Chang, H.S., Wang, X. and Quail, P.H. (2001) Multiple transcription-factor genes are early targets of phytochrome A signaling. Proc. Natl Acad. Sci. USA 98: 9437–9442.

Van Leene J, Witters E, Inzé D, De Jaeger G (2008) Boosting tandem affinity purification of plant protein complexes. Trends Plant Sci 13: 517–520.

Van Leene, J. *et al*. (2015). An improved toolbox to unravel the plant cellular machinery by tandem affinity purification of Arabidopsis protein complexes. Nature protocols 10, 169-187, doi:10.1038/nprot.2014.199.

Van Leene, J. *et al*. 2011. Isolation of transcription factor complexes from Arabidopsis cell suspension cultures by tandem affinity purification. Methods in molecular biology 754, 195-218.

van Zanten M, Tessadori F, McLoughlin F, Smith R, Millenaar FF, van Driel R, Voesenek LA, Peeters AJ, Fransz P. 2010. Photoreceptors CRYTOCHROME2 and phytochrome B control chromatin compaction in Arabidopsis. Plant Physiology 154, 1686–1696.

Vierstra, R.D. (2009) The ubiquitin–26S proteasome system at the nexus of plant biology. Nat. Rev. Mol. Cell Biol. 10, 385–397.

Vlachonasios KE Thomashow MF Triezenberg SJ. 2003. Disruption mutations of ADA2b and GCN5 transcriptional adaptor genes dramatically affect Arabidopsis growth, development, and gene expression. The Plant Cell 15, 626–638.

Voinnet O, Rivas S, Mestre P, Baulcombe D. (2003).An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J. 33(5):949-56.

von Arnim, A.G., Osterlund, M.T., Kwok, S.F., Deng, X.W. (1997). Genetic and developmental control of nuclear accumulation of COP1, a repressor of photomorphogenesis in Arabidopsis. Plant Physiology 114:779-788.

Wang L., Dent S. Y. (2014). Functions of SAGA in development and disease. Epigenomics 6, 329–339.

Wang, X., Li, W., Piqueras, R., Cao, K., Deng, X.W., Wei, N. (2009). Regulation of COP1 nuclear localization by the COP9 signalosome via direct interaction with CSN1. ThePlant Journal 58:655-667.

Weake V. M., Workman J. L. (2008). Histone ubiquitination: triggering gene activity. *Mol. Cell* 29653–663.

Weake V. M., Workman J. L. (2012). SAGA function in tissue-specific gene expression. Trends Cell Biol. 2012 Apr; 22(4): 177–184.

Wei N, Deng XW (1996) The role of the COP/DET/FUS genes in light control of Arabidopsis seedling development. Plant Physiol 112:871–878.

Wei N. & Deng X.-W. (1996) The role of the COP/DET/FUS genes in light control of Arabidopsis seedling development. Plant Physiology,112. 871-878.

Wei, N., Tsuge, T., Serino, G., Dohmae, N., Takio, K., Matsui, M., and Deng, X.W. (1998). The COP9 complex is conserved between plants and mammals and is related to the 26S proteasome regulatory complex. Curr. Biol. 8, 919–922.

Weigel, D., and Glazebrook, J. (2002). Arabidopsis: A Laboratory Manual. CSHL Press.

Wilkinson C.R., Seeger M., Hartmann-Petersen R., Stone M., Wallace M., Semple C., Gordon C. (2001). Proteins containing the UBA domain are able to bind to multi-ubiquitin chains. Nat. Cell Biol.3: 939–943.

Woodcock, D. M. *et al*. Quantitative evaluation of Escherichia coli host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic acids research 17, 3469-3478 (1989).

Wu P. Y., Ruhlmann C., Winston F., Schultz P. (2004). Molecular architecture of the *S. cerevisiae* SAGA complex. Mol. Cell. ;15:199–208.

Xin H., Takahata S., Blanksma M., McCullough L., Stillman D. J., *et al*. , (2009). yFACT induces global accessibility of nucleosomal DNA without H2A–H2B displacement. Mol. Cell 35: 365–376.

Xu P., Xiang Y., Zhu H., Xu H., Zhang Z., Zhang C., Zhang L., Ma Z. (2009). Wheat cryptochromes: Subcellular localization and involvement in photomorphogenesis and osmotic stress responses. Plant Physiol. 149: 760–774.

Xu, X., Paik, I., Zhu, L., Bu, Q., Huang, X., Deng, X.W., Huq, E. (2014). PHYTOCHROME INTERACTING FACTOR1 enhances the E3 ligase activity of CONSTITUTIVE PHOTOMORPHOGENIC1 to synergistically repress photomorphogenesis in Arabidopsis. Plant Cell 26:1992-2006.

Yanagawa, Y., Sullivan, J.A., Komatsu, S., Gusmaroli, G., Suzuki, G., Yin, J., Ishibashi, T., Saijo, Y., Rubio, V., Kimura, S., Wang, J., Deng, X.W. (2004). Arabidopsis COP10 forms a complex

with DDB1 and DET1 in vivo and enhances the activity of ubiquitin conjugating enzymes. Genes and Development 18:2172-2181.

Yang J, Lin R, Sullivan J, Hoecker U, Liu B, Xu L, et al. (2005). Light regulates COP1-mediated degradation of HFR1, a transcription factor essential for light signaling in Arabidopsis. Plant Cell.;17:804–821.

Yang J., Lin R., Sullivan J., Hoecker U., Liu B., Xu L., Deng X.W., Wang H., *et al*. (2005). Light regulates COP1-mediated degradation of HFR1, a transcription factor essential for light signaling in Arabidopsis. Plant Cell.;17:804–821.

Yi, C., Deng, X.W. (2005). COP1–from plant photomorphogenesis to mammalian tumorigenesis. Trends in Cell Biology 15:618-625.

Yu J.W., Rubio V., Lee N.Y., Bai S., Lee S.Y., Kim S.S., Liu L., Zhang Y., Irigoyen M.L., Sullivan J.A., Zhang Y., Lee I., Xie Q., Paek N.C., Deng X.W. (2008). COP1 and ELF3 control circadian function and photoperiodic flowering by regulating GI stability. Mol. Cell. 32:617–630.

Yu X., Liu H., Klejnot J., Lin C. (2010). The Cryptochrome Blue Light Receptors. The Arabidopsis Book.8:e0135.

Zhang X., Germann S., Blus BJ., Khorasanizadeh S., Gaudin V., and Jacobsen SE. (2007). The Arabidopsis LHP1 protein colocalizes with histone H3 Lys27 trimethylation. Nature Structural and Molecular Biology 14, 869–871.

Zhang Y. (2003). Transcriptional regulation by histone ubiquitination and deubiquitination. Genes and Development 17, 2733–2740.

Zhang, X. Y., M. Varthi, S. M. Sykes, C. Phillips, C. Warzecha, W. Zhu, A. Wyce, A. W. Thorne, S. L. Berger, and S. B. McMahon. (2008) The putative cancer stem cell marker USP22 is a subunit of the human SAGA complex required for activated transcription and cell-cycle progression. Mol Cell 29: 102–111.

Zuo, Z., Liu, H., Liu, B., Liu, X., Lin, C. (2011). Blue light-dependent interaction of CRY2 with SPA1 regulates COP1 activity and floral initiation in Arabidopsis.Curr. Biol. 21: 841–847.

APPENDIX

APPENDIX I.

Resumen.

Las plantas adaptan su crecimiento y desarrollo en función de la luz ambiental. Las señales luminosas son percibidas, procesadas e integradas a través de redes intracelulares altamente estructuradas. Esto ocurre gracias a una precisa regulación transcripcional mediada por procesos de remodelación de la cromatina sincronizados por la unión de factores de transcripción al ADN, necesitándose un control estricto de su abundancia y actividad. El control de los niveles de los factores de transcripción está mediado, en muchos casos, por procesos específicos de ubiquitinación y degradación por el proteosoma 26S. La ubiquitinación de las proteínas diana se lleva a cabo, entre otros, por ubiquitin ligasas de tipo Cullin 4 E3 (CRL4s) en asociación con complejos de tipo COP10-DDB1-DET1-DDA1 (CDDD). Recientemente hemos caracterizado a DDA1 como un nuevo tipo de adaptador de substrato para los complejos CRL4-CDDD (Irigoyen *el al.,* 2014). Mediante un análisis de doble híbrido, se pudo determinar que SGF11 era un interactor de DDA1. SGF11 es un miembro de un módulo de desubiquitinación (DUBm) que, tanto en levaduras como en animales, ha mostrado formar parte del complejo SAGA (SPT-ADA-GCN5-Acetiltransferasa). El papel de DUBm desubiquitinando a H2Bub (histona 2B ubiquitinada) para promover la elongación de la transcripción está bien descrito en levaduras, *Drosophila* y mamíferos. Sin embargo, en plantas la función de DUBm está aún por explorar.

En este estudio mostramos que el módulo DUBm de *Arabidopsis* está compuesto por, al menos, tres subunidades: SGF11, ENY2 y UBP22, siendo esta última la proteasa que actúa de forma específica sobre H2B modificada con ubiquitina. En concordancia con lo predicho en experimentos *in silico*, la pérdida de función de SGF11 o UBP22 deriva en un aumento de la abundancia de H2Bub. La función de DUBm está prácticamente controlada por las condiciones de luz ya que los tres componentes del módulo son degradados en condiciones de oscuridad. Hemos podido demostrar que la degradación de SGF11 precisa de la función de DET1, lo que sugiere que, tras el reconocimiento de SGF11 por DDA1, las CRL4-CDDD E3 ligasas promueven su ubiquitinación y su posterior degradación por el proteosoma. De acuerdo con esto, la función de CRL4-CDDD altera la abundancia global de H2Bub.

La caracterización fenotípica de los mutantes *sgf11* y *ubp22* muestra que DUBm también juega un papel en el control de la respuesta de las plantas a la luz. En consecuencia, ambos

mutantes *sgf11* y *ubp22* realzan de manera sinérgica el fenotipo detiolado que muestran las plantas mutantes *det1-1* en condiciones de oscuridad, mientras que la sobreexpresión de SGF11 mejora los defectos fotomorfogénicos de *det1-1*. Estos defectos fotomorfogénicos se correlacionan, respectivamente, con un aumento o reducción de la abundancia de HY5, un regulador positivo de la respuesta a la luz cuya acumulación se encuentra reprimida por las CRL4-CDDD E3 ligasas. En conclusión, estos resultados indican que DUBm actúa como un regulador negativo de la fotomorfogénesis, y apuntan a un papel importante de la desubiquitinación de H2B en este proceso. Proponemos un modelo en el que DUBm facilita el reclutamiento de los complejos CRL4-CDDD hacia regiones promotoras de respuesta a luz con el fin de aumentar el reconocimiento y la degradación de HY5 y así reprimir el desarrollo fotomorfogénico.

Objetivos:

Previamente a este estudio no había ninguna evidencia acerca de la función de DUBm en plantas. El hecho de que SGF11 interacciona con DDA1, un componente de las CRL4-CDDD E3 ligasas, sugiere un posible vínculo entre la maquinaria de remodelado de cromatina y el sistema de ubiquitina-proteasoma que podría tener un impacto en el crecimiento y desarrollo de la planta derivado del cambio de las condiciones de luz. Esta idea nos llevó a investigar la relevancia funcional y fisiológica de la interacción DUBm/CRL4-CDDD. Con este propósito, nos propusimos los siguientes objetivos:

1. Caracterización bioquímica del complejo DUBm y su asociación con las CRL4-CDDD E3 ligasas.
2. Caracterización funcional del complejo DUBm de Arabidopsis.
3. Estudio del efecto de las CRL4-CDDD E3 ligasas sobre la estabilidad de los componentes de DUBm y sobre la abundancia de H2B ubiquitinado (H2Bub).
4. Caracterización de los efectos de DUBm en el control de la fotomorfogénesis mediado por CRL4-CDDD.

Conclusiones.

1. UBP22, SGF11 y ENY2 son componentes del complejo DUBm complex en *Arabidopsis*, el cual está implicado en la desubiquitinación de H2Bub.
2. UBP22, SGF11, DET1 y DDA1 se localizan en el núcleo, asociados a eucromatina transcripcionalmente activa, mientras que ENY2 se encuentra presente también en regiones heterocromáticas.
3. SGF11 es una diana de la ubiquitinación mediada por CRL4-CDDD y se degrada en el proteosoma 26S.
4. Las condiciones lumínicas controlan la abundancia del complejo DUBm ya que SGF11, UBP22 y ENY2 se degradan en el proteosoma 26S preferentemente en la oscuridad.
5. Alteraciones en la función de DET1 y DDA1 afectan a los niveles globales de las marcas de histona H2Bub, H3K36me3 y H3K4me3, lo que indica un papel de los complejos CRL4-CDDD en la regulación de la maquinaria epigenética. El efecto de CRL4-CDDD sobre las modificaciones de la cromatina está muy probablemente asociado a su actividad en el control de la abundancia de SGF11.
6. La pérdida de función de SGF11 o UBP22 produce un efecto sinérgico sobre los defectos fotomorfogénicos asociados a la mutación *det1*, Dicho efecto sinérgico correlaciona con la abundancia del factor transcripcional HY5 en las líneas correspondientes. Este hecho sugiere que el complejo DUBm facilita la represión mediada por CRL4-CDDD de la señalización lumínica.
7. La sobreexpresión de SGF11 suprime parcialmente los defectos fenotípicos de los mutantes *det1,* lo que sugiere un papel de SGF11 en el reclutamiento de los complejos CRL4-CDDD a la cromatina, en el reconocimiento de HY5, y en la represión de la expresión génica en respuesta a la luz.