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DELLA proteins restrain germination and elongation growth in *Arabidopsis thaliana* COP9 signalosome mutants

Esther Mirjam Natascha Dohmann [a,1], Carola Nill [a], Claus Schwechheimer [a,b,*]

[a] Department of Developmental Genetics, Center for Plant Molecular Biology, Tübingen University, Auf der Morgenstelle 5, 72076 Tübingen, Germany
[b] Department of Plant Systems Biology, Technische Universität München, Am Hochanger 4, 85354 Freising-Weihenstephan, Germany

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ABSTRACT

The COP9 signalosome (CSN) is an evolutionarily conserved multiprotein complex with an essential role in the development of higher eukaryotes. CSN deconjugates the ubiquitin-related modifier NEDD8 from the cullin subunit of cullin-RING type E3 ubiquitin ligases (CRLs), and CSN-mediated cullin deneddylation is required for full CRL activity. Although several plant E3 CRL functions have been shown to be compromised in *Arabidopsis csn* mutants, none of these functions have so far been shown to limit growth in these mutants. Here, we examine the role of CSN in the context of the E3 ubiquitin ligase $SCF^{SLEEPY1\ (SLY1)}$, which promotes gibberellic acid (GA)-dependent responses in *Arabidopsis thaliana*. We show that *csn* mutants are impaired in GA- and SCF^{SLY1}-dependent germination and elongation growth, and we show that these defects correlate with an accumulation and reduced turnover of an SCF^{SLY1}-degradation target, the DELLA protein REPRESSOR-OF-*ga1*-3 (RGA). Genetic interaction studies between *csn* mutants and loss-of-function alleles of *RGA* and its functional homologue *GIBBERELLIC ACID INSENSITIVE* (*GAI*) further reveal that RGA and GAI repress defects of germination in strong *csn* mutants. In addition, we find that these two DELLA proteins are largely responsible for the elongation defects of a weak *csn5* mutant allele. We thus conclude that an impairment of SCF^{SLY1} is at least in part causative for the germination and elongation defects of *csn* mutants, and suggest that DELLA proteins are major growth repressors in these mutants.

Introduction

The COP9 signalosome (CSN) is an eight-subunit protein complex that was originally described in plants following the identification of the *CONSTITUTIVE PHOTOMORPHOGENIC9* (*COP9*) gene from *Arabidopsis* (Chamovitz et al., 1996; Wei et al., 1994). *Arabidopsis* mutants of *COP9* (*CSN SUBUNIT8, CSN8*) and any of the seven other CSN subunits (*CSN1* – *CSN7*) have an identical morphological and – as far as can be judged today – molecular phenotype, suggesting that the loss of a single CSN subunit fully impairs CSN function (Dohmann et al., 2005, 2008a; Gusmaroli et al., 2007; Kwok et al., 1996). Based on the physiological and molecular analysis of mutant phenotypes, *Arabidopsis* CSN has so far been shown to repress photomorphogenesis in dark-grown seedlings, to promote responses to the phytohormones auxin and jasmonic acid, and to ensure proper floral development, cell cycle progression as well as genomic stability (Chen et al., 2006; Dohmann et al., 2008a, b; Feng et al., 2003; Schwechheimer et al., 2001, 2002; Wang et al., 2003; Yanagawa et al., 2004). CSN is conserved in all eukaryotes and has been implicated in a broad set of cellular functions in yeasts and animals, ranging from the control of cell cycle progression to neural development (Richardson and Zundel, 2005; Suh et al., 2002; Wei et al., 2008).

A major aspect of the cellular function of CSN appears to reside in its ability to interact with cullin-RING-type E3 ubiquitin ligases (CRLs) and to promote the deconjugation of the ubiquitin-related modifier NEDD8 from their cullin subunit via the deneddylation activity of CSN5 (Cope et al., 2002; Lyapina et al., 2001; Schwechheimer et al., 2001). CRLs specifically recognize proteins that are destined for degradation by the 26S proteasome and they promote the poly-ubiquitylation of degradation substrates by E2 ubiquitin-conjugating enzymes (Bosu and Kipreos, 2008; Schwechheimer and Calderon Villalobos, 2004). Conjugation of the ubiquitin-related protein NEDD8 to the CRL cullin subunit is a posttranslational modification of cullins, and NEDD8 conjugation as well as CSN-mediated NEDD8 deconjugation are required for full E3 ligase activity (Dohmann et al., 2008b; Hotton and Callis, 2008). In plants, three types of CRLs have been reported including SCF (SKP1/CDC53/F-BOX PROTEIN)-type complexes that consist of the core subunits cullin1, RING BOX1 (RBX1), and SKP1 in addition to an interchangeable F-box protein subunit (Gray et al.,

* Corresponding author at: Department of Plant Systems Biology, Technische Universität München, Am Hochanger 4, 85354 Freising-Weihenstephan, Germany. Tel.: +49 8161 712 880; fax: +49 8161 712 886.
E-mail address: claus.schwechheimer@wzw.tum.de (C. Schwechheimer).
[1] Present address: Department of Plant Molecular Biology, University of Lausanne, 1015 Lausanne, Switzerland.

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1999; Schwechheimer and Calderon Villalobos, 2004). F-box proteins are the degradation target receptor subunits of SCF complexes and most if not all of the 700 F-box proteins encoded by the *Arabidopsis* genome are expected to form SCF complexes with distinct substrate specificities (Gagne et al., 2002). It has been observed that F-box protein/degradation target complexes can trigger the formation of SCF complexes and subsequently cullin neddylation (Bornstein et al., 2006; Chew and Hagen, 2007). In turn, it has been hypothesized that CSN-mediated deneddylation promotes complex disassembly and that CSN thereby contributes to the de novo formation and stability of SCF-type CRLs.

The CSN–CRL interaction and its biological relevance were first identified by examining the role of CSN in $SCF^{TRANSPORT\ INHIBITOR\ RESISTANT1\ (TIR1)}$-mediated auxin responses (Schwechheimer et al., 2001). Since CSN physically interacts with the SCF core subunits cullin1 and RBX1, it is assumed that CSN interacts with all SCF-type E3s. To date, biochemical and functional interactions between CSN and $SCF^{CORONATINE\ INSENSITIVE1\ (COI1)}$ (jasmonic acid responses) and $SCF^{UNUSUAL\ FLORAL\ ORGANS\ (UFO)}$ (floral development) have been reported (Feng et al., 2003; Schwechheimer et al., 2002; Wang et al., 2003). Based on genetic and biochemical evidence it is also established that CSN interacts with a cullin4-containing CRL that represses photomorphogenesis together with the proteins COP1, DET1 and SPA1 (Chen et al., 2006; Fittinghoff et al., 2006; Saijo et al., 2008; Zhang et al., 2008).

Although CSN has been implicated in individual SCF-mediated processes, none of these processes has so far been shown to be critical for *csn* mutant growth. $SCF^{SLY1(SLEEPY1)}$ is a plant-specific CRL that promotes the gibberellic acid (GA)-dependent ubiquitylation and proteasomal degradation of DELLA repressor proteins such as *REPRESSOR OF ga1-3* (*RGA*) and *GIBBERELLIC ACID INSENSITIVE* (*GAI*) (Dill et al., 2004; Fu et al., 2004; McGinnis et al., 2003). SCF^{SLY1} activity is important for a number of GA-regulated growth responses such as seed germination, elongation growth, and flowering time control. Here, we examine the role of CSN in SCF^{SLY1}-dependent growth processes. We find that *csn* mutants have reduced GA-sensitivity and fail to efficiently degrade the SCF^{SLY1}-degradation substrate RGA. We find furthermore that the loss of RGA and GAI function suppresses at least partially the germination defect of a strong *csn* mutant and the semi-dwarfism of an intermediate *csn5a* mutant allele. Taken together, our data suggest that the impairment of SCF^{SLY1} function is critical for some important aspects of *csn* mutant growth.

Materials and methods

Biological material

Arabidopsis mutants were grown on soil or on Murashige & Skoog medium supplemented with 1% sucrose in standard growth conditions under continuous light. The following mutant alleles were used for the studies presented in this work: *csn3*/*fus11*-U203 (*Ler*), *csn5a*-1 (Col), *csn5a*-2 (Col), *csn5b*-1 (Col), *csn5a*-2 *csn5b*-1 (Col), *csn8*/*fus8*-S253 (*Ler*), *ga1*-3 (*Ler*), *gai*-t6 *rga*-24 (*Ler*), *sly1*-10 (*Ler*) (Dohmann et al., 2005; King et al., 2001; Kwok et al., 1996; McGinnis et al., 2003). *csn*, gai-t6, and *rga*-24 mutations were identified by PCR-based genotyping as described previously (Dohmann et al., 2008a; Willige et al., 2007). To generate *csn5a*-2 *gai*-t6, *csn5a*-2 *rga*-24 and *csn5a*-2 *gai*-t6 *rga*-24 mutants, *csn5a*-2 (Col) was crossed to the *gai*-t6 *rga*-24 (*Ler*) double mutant. *csn5a*-2 *GAI*/*gai*-t6 *RGA*/*rga*-24 plants were identified by genotyping and backcrossed three times to *csn5a*-2. In the F2, the *csn5a*-2 *gai*-t6, *csn5a*-2 *rga*-24 and *csn5a*-2 *gai*-t6 *rga*-24 double and triple mutants were identified and analyzed. To obtain *csn8 gai*-t6 *rga*-24, *csn8*/*fus8*-S253 (*Ler*) mutants were crossed to a *gai*-t6 *rga*-24 double mutant and *CSN8*/*csn8* gai-t6 *rga*-24 plants were identified in the F2 generation. F3 progeny was then analyzed with regard to germination and hypocotyl elongation as described below.

Plant growth conditions

To analyze germination, at least 2-week-old seeds were surface-sterilized and plated on Murashige & Skoog medium supplemented with 1% sucrose. Following stratification for 7 days (Fig. 1) or 2 days (Figs. 3 and 4) at 4 °C, seeds were transferred to standard growth conditions, and germination (radicle emergence) was monitored at various time points as specified in the figures. Germination experiments with *csn* mutants were performed with the progeny of *csn* +/- parent lines. Following germination, homozygous mutants were identified based on the *csn* mutant phenotype and germination rates were determined specifically for this subpopulation. Hypocotyl elongation was quantified from 7-day-old dark- or light-grown seedlings using ImageJ64 (National Institutes of Health, Bethesda, MD). To analyze the effect of GA on mature plants, plants were sprayed with different concentrations of GA3 (Duchefa, Harlem, The Netherlands), and rosette diameters were measured from 35-day-old plants.

Immunoblots

Immunoblots with the anti-RGA antibody were carried out as previously described (Willige et al., 2007). To analyze the GA-dependent degradation of RGA, 7-day-old seedlings were treated with 100 μM GA3 in liquid Murashige & Skoog medium for different time points as specified in the figure and immediately frozen in liquid nitrogen for subsequent protein extraction and Western blot analysis.

Results

csn mutants are GA-insensitive

In *Arabidopsis*, $SCF^{SLEEPY1(SLY1)}$ mediates the GA-dependent degradation of the DELLA repressors RGA, GAI, and RGA-LIKE1-3 (RGL1-3). SCF^{SLY1} thereby promotes GA-dependent growth processes such as germination, elongation growth, and the onset of flowering (Dill et al., 2004; Fu et al., 2004). Mutants deficient in the pathway-specific F-box protein SLY1 as well as mutants deficient in GA biosynthesis (*gibberellic acid1*, *ga1*) or in GA-receptor function (*gibberellic acid insensitive dwarf1a-c*, *gid1a-c*) are partially (*sly1*) or fully (*ga1*, *gid1a-c*) defective in germination, cell expansion, and elongation growth (Dill et al., 2004; Fu et al., 2004; McGinnis et al., 2003).

Since *Arabidopsis* CSN had previously been shown to be required for the full activity of SCF-type E3s, we were interested in examining the role of CSN in regard to SCF^{SLY1} function and GA signaling (Dohmann et al., 2008b; Schwechheimer et al., 2001). We therefore tested germination and GA-promoted elongation growth in the absence and presence of exogenous GA in *csn* loss-of-function mutants (*csn3/fus11*, *csn5ab*, and *csn8/fus8*) as well as in *csn5a* and *csn5b* mutants (Dohmann et al., 2005; Gusmaroli et al., 2004). The *csn3*, *csn5ab*, and *csn8* mutants chosen for this study are strong mutant alleles that arrest growth at the seedling stage and that are fully deficient in cullin deneddylation (Dohmann et al., 2005, 2008b; Gusmaroli et al., 2004). The *csn5a*-2 and *csn5b*-1 mutants are deficient in one of the two *CSN5* genes from *Arabidopsis*; the *csn5a*-2 and *csn5b*-1 alleles are viable and fertile intermediate (*csn5a*) and weak (*csn5b*)

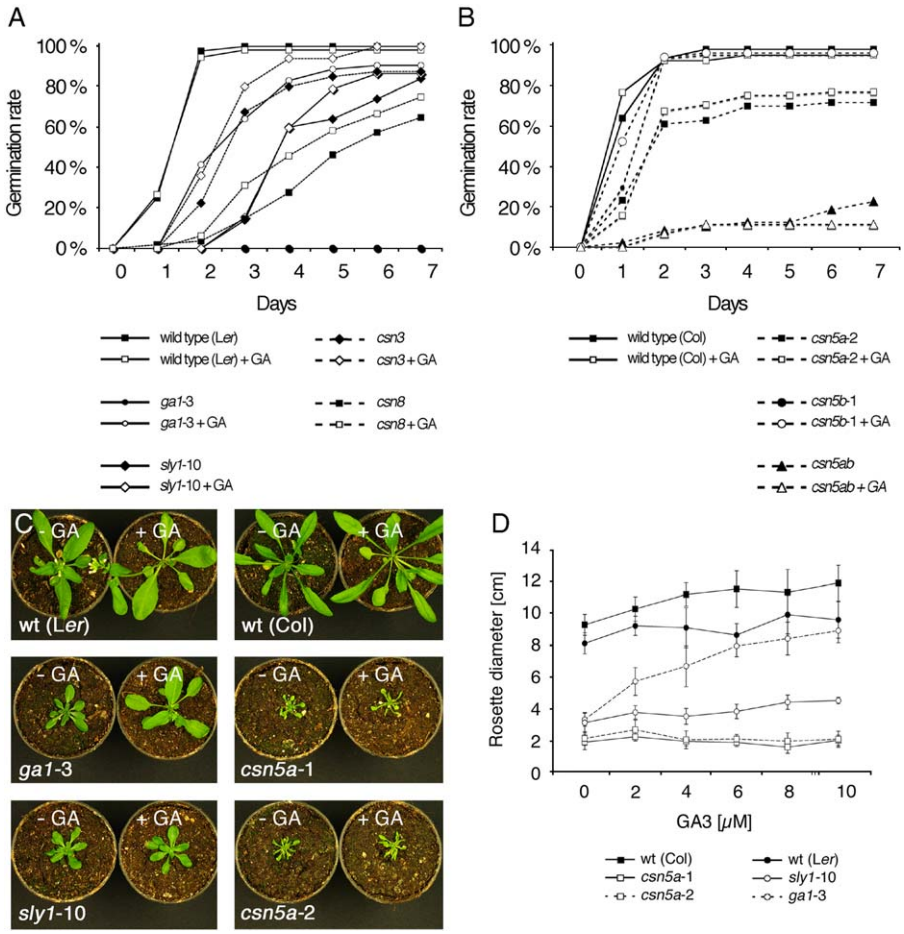

Fig. 1. *csn* mutants are GA insensitive. (A, B) Germination rate of specific csn mutant alleles in comparison to the GA biosynthesis mutant ga1-3 and the GA signaling mutant sly1-10 in the Landsberg erecta (Ler) and Columbia (Col) backgrounds, respectively. (C) Rosette phenotype of 4-week-old wild-type and mutant plants that were sprayed once per week with 10 μM GA3. (D) Quantification of the rosette diameter of 5-week-old plants sprayed once per week with different concentrations of GA3. n=10. Genotypes are indicated in the figure.

mutants, respectively, that are partially (*csn5a*) or only mildly (*csn5b*) defective in cullin deneddylation (Dohmann et al., 2005; Gusmaroli et al., 2004).

When we examined the germination rate of strong *csn* mutants, we measured a significant germination delay in the strong *csn* mutants when compared to the respective Landsberg *erecta* (*Ler*) and Columbia (Col) wild types (Fig. 1A, B): We found that only a fraction of the *csn3*, *csn5ab* and *csn8* mutant seeds had germinated after 2 days and that full germination was not reached even after 7 days, while all wild-type seeds had germinated at day 2 of the experiment. The germination defect was less pronounced in the intermediate *csn5a*-2 mutant and only marginal in the weak *csn5b*-1 mutant. While the addition of GA could strongly promote germination in the otherwise germination-deficient *ga1*-3 mutant, its effect on the germination rate of the strong *csn* mutants was comparatively minor. Thus, the *csn* mutants behave similarly to the germination-impaired and GA-insensitive *sly1* mutant, and we conclude that the *csn* mutants are impaired in germination.

The two available intermediate *csn5a* mutant alleles are semi-dwarfed and have smaller rosette leaves than the wild type. In that respect, *csn5* mutants are similar to GA mutants such as *ga1*-3 and *sly1* (Fig. 1C, D). To elucidate whether the semi-dwarfism of *csn* mutants may be attributed to reduced GA responses, we examined rosette leaf expansion in the absence and presence of GA in these different mutants (Fig. 1C, D). Unlike the rosette leaf phenotype of *ga1*-3 mutants, which was efficiently normalized by regular GA application, we observed no effect of the GA treatments on the *csn5a* mutants. While even wild-type rosette leaves responded at least to some extent to GA treatments, the *csn* mutants were GA-insensitive, similarly to *sly1* mutants. We thus conclude that *csn* mutants are defective in GA-promoted growth processes.

The DELLA repressor RGA is stabilized in csn mutants

The germination and elongation defects of *csn* mutants may be the result of a defect in the GA- and SCF^{SLY1}-dependent turnover of DELLA repressors such as RGA. We therefore examined RGA accumulation in the wild type and in *csn* mutant backgrounds (Willige et al., 2007). Indeed, this study revealed a strong accumulation of RGA in *csn3*, *csn5ab*, *csn8* as well as in the intermediate *csn5a* mutants (Fig. 2A). In turn, we found RGA levels to be indistinguishable between the wild type and the weak *csn5b* mutants. Thus, RGA accumulates in *csn* mutants and its accumulation correlates well with the severity of the *csn* mutant phenotype.

We next tested whether the increased abundance of RGA is a consequence of reduced CSN and SCF^{SLY1}-dependent degradation. Therefore, we examined the GA-induced turnover of RGA in a subset of *csn* mutants. In these experiments, we observed a significant degradation of RGA in wild-type seedlings already 5 min following GA application (Fig. 2B). At the same time, RGA

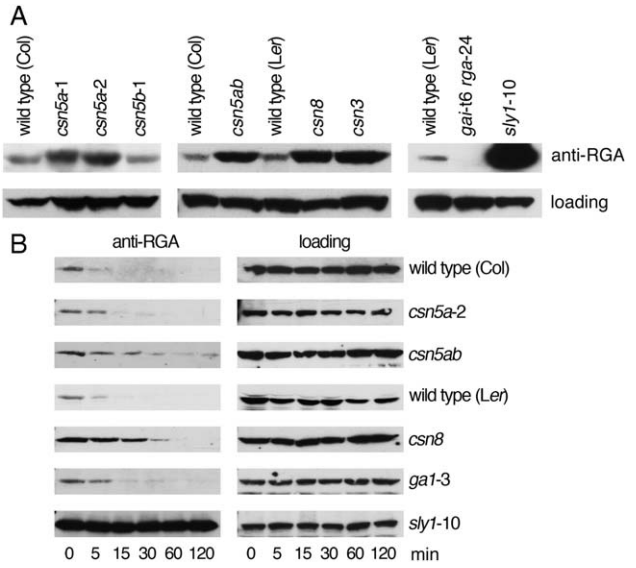

Fig. 2. The degradation of the SCF^{SLY1}-target RGA is impaired in *csn* mutants. (A) Western blot analysis with an anti-RGA antibody reveals increased basal levels of RGA in various *csn* mutants. (B) Western blot analysis with an anti-RGA antibody reveals a decreased protein turnover in GA-treated *csn* mutants. Genotypes are as specified in the figure. A cross-reacting band serves as loading control in both experiments.

degradation was strongly reduced in *csn5a* mutants and even more reduced in *csn5ab* and *csn8* (Fig. 2B). In line with previous reports, RGA was fully stabilized in the *sly1* mutant (Fig. 2B). We thus conclude that the loss of CSN function causes impaired RGA degradation, which may result in increased RGA levels. At the same time, these data also show that CSN promotes but is not essential for the GA-dependent degradation of this DELLA repressor. SCF^{SLY1} function, in turn, appears to be essential for this degradation event.

DELLA proteins are critical repressors of GA responses in csn mutants

To date, four of the five *Arabidopsis* DELLA proteins (RGA, GAI, RGL1 and RGL2) have been implicated in different GA-dependent responses based on their ability to suppress different aspects of the phenotypes of the *ga1*-3 GA biosynthesis mutant, the *gid1a-c* GA receptor mutant, or the *sly1*-10 F-box protein mutant (Dill and Sun, 2001; Dill et al., 2004; Fu et al., 2004; King et al., 2001; Lee et al., 2002; Schwechheimer et al., 2009; Willige et al., 2007). These studies have shown that RGA and GAI act redundantly as repressors of elongation growth of GA biosynthesis and GA signaling mutants, and that the two proteins must act together with RGL2 in the control of GA-dependent germination (Lee et al., 2002; Willige et al., 2007). In order to assess the contribution of RGA and GAI to the GA-insensitivity phenotype of the *csn* loss-of-function mutants, we introduced the *RGA* and *GAI* loss-of-function alleles *rga*-24 *and* gai-*t6* into the strong *csn8* mutant background (Fig. 3A). We subsequently established a *csn8*/*CSN8 rga*-24/*rga*-24 *gai*-t6/*gai*-t6 line and examined homozygous *csn8*/*csn8 rga*-24/*rga*-24 *gai*-t6/*gai*-t6 mutants in comparison to *csn8* and *rga*-24 *gai*-t6 mutants in regard to seed germination and hypocotyl elongation. Interestingly, the *csn8 rga*-24 *gai*-t6 triple mutant germinated significantly better than the *csn8* single mutant, suggesting that RGA and GAI exert an important repressor function in the *csn*8 mutant (Fig. 3B). In comparison, loss of *RGA* and *GAI* resulted only in a marginal suppression of the *csn8* hypocotyl phenotype in dark- as well as in light-grown seedlings (Fig. 3C, D). Taken together, these data suggest that DELLA proteins are critical repressors of germination but not of hypocotyl elongation in *csn8* mutants.

We next performed a genetic analysis to examine the role of *RGA* and *GAI* as potential growth repressors during germination, hypocotyl elongation, and adult mutant growth in the intermediate *csn5a*-2 mutants. Since the *csn5a*-2 and the *rga*-24 *gai*-t6 mutants are in different ecotypes (Col and L*er*, respectively), we back-crossed a *csn5a*-2 *gai*-t6/*GAI rga*-24/*RGA* mutant resulting from this cross three times to *csn5a*-2 (Col) to minimize the effect of the Landsberg *erecta* genome. We then identified *csn5a*-2 *gai*-t6 as well as *csn5a*-2 *rga*-24 double mutants from these backcrosses. Since we found the *csn5a*-2 *gai*-t6 *rga*-24 triple mutant to be infertile, homozygous triple mutants were isolated by genotyping the segregating progeny of a *csn5a*-2 *gai*-t6 *rga*-24/*RGA* mutant for physiological experiments. The analysis of the different mutant backgrounds revealed a gradual suppression of the moderate germination defect of the *csn5a*-2 mutant, which was most pronounced in *csn5a* mutants lacking both DELLA proteins, less pronounced in mutants lacking *RGA* and least pronounced in mutants lacking *GAI* (Fig. 4A). Besides a partial suppression of the hypocotyl elongation defect of dark-grown *csn5a*-2 mutants, this analysis most importantly revealed an almost complete suppression of the elongation defect of the adult *csn5a* mutant. In adult *csn5a*-2 mutants, the genetic suppression was most pronounced in the *csn5a*-2 *rga*-24 and the *csn5a*-2 *gai*-t6 *rga*-24 mutants (Fig. 4B-D). In this respect, this analysis is in line with previously published genetic interactions that had revealed that RGA is a more dominant growth repressor than GAI in GA-signaling or biosynthesis mutants. In summary, our genetic experiments show that the DELLA proteins RGA and GAI are important repressors of germination in *csn* mutants and that they restrict elongation growth in seedlings and adult plants of weak *csn* mutants. We thus conclude that DELLA protein accumulation is at least in part responsible for the *csn* mutant phenotype.

Discussion

In the present study, we have examined the proposed role of CSN in SCF^{SLY1}-dependent GA-responses. We found that GA-responses are impaired in *csn* mutants and that the severity of this impairment correlates with the degree of loss of CSN function (and cullin deneddylation) in different *csn* mutant alleles. The impairment of GA-responses also correlates with increases in the abundance of the SCF^{SLY1} degradation target RGA, which we have shown to be a result of reduced RGA turnover. Furthermore, we provide genetic evidence that the SCF^{SLY1}-degradation targets RGA and GAI restrict germination in *csn* mutant seeds and that these two proteins limit elongation growth in an intermediate *csn5a* mutant. To our knowledge we have thus identified the first E3 ubiquitin ligase degradation target, the accumulation of which restricts growth in *Arabidopsis csn* mutants.

The use of *RGA* and *GAI* loss-of-function mutants has allowed to show in a number of genetic studies that RGA and GAI differentially contribute to growth repression in GA biosynthesis and signaling mutants (Dill and Sun, 2001; Dill et al., 2004; Fu et al., 2004; King et al., 2001; Lee et al., 2002; Schwechheimer et al., 2009; Willige et al., 2007). In each case, the respective contribution of RGA to the growth repression was more pronounced than that of GAI. We observed a similar genetic interaction in *csn* mutants where the repression mediated by RGA was more pronounced than that by GAI. The fact that only a partial but not a full suppression of the germination defect was achieved at least in the severe *csn* mutants could be attributed to the repression by other DELLA proteins such as RGL2, which may

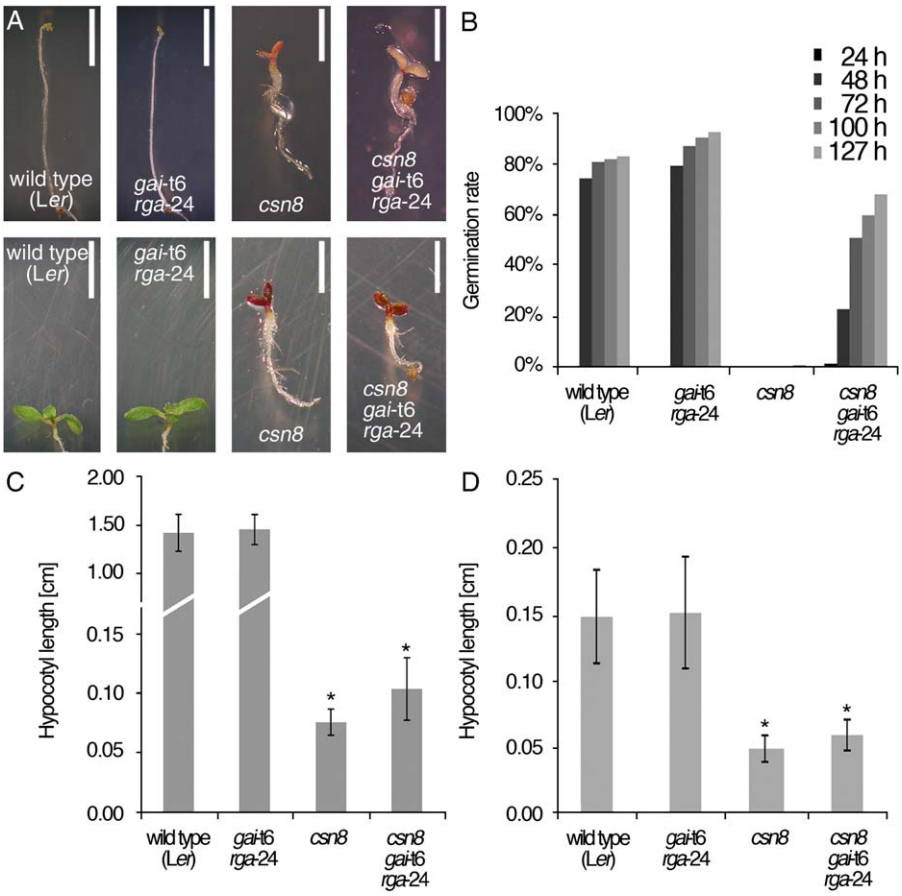

Fig. 3. RGA and GAI repress germination in *csn8* mutants. (A) Phenotypes of dark-grown (upper row) and light-grown (lower row) wild-type and mutant seedlings as specified in the figure. Scale bar (wild type, *gai*-t6 *rga*-24)=5 mm; scale bar (*csn8*, *csn8 gai*-t6 *rga*-24)=1 mm. (B) Germination rate of wild-type and *csn8* mutant seed as specified in the figure. $n \geq 85$. (C) Hypocotyl length of 7-day-old dark-grown and (D) light-grown wild-type and mutant seedlings. $n \geq 10$. The asterisk (∗) indicates a significant difference ($p < 0.005$; Student's t-test).

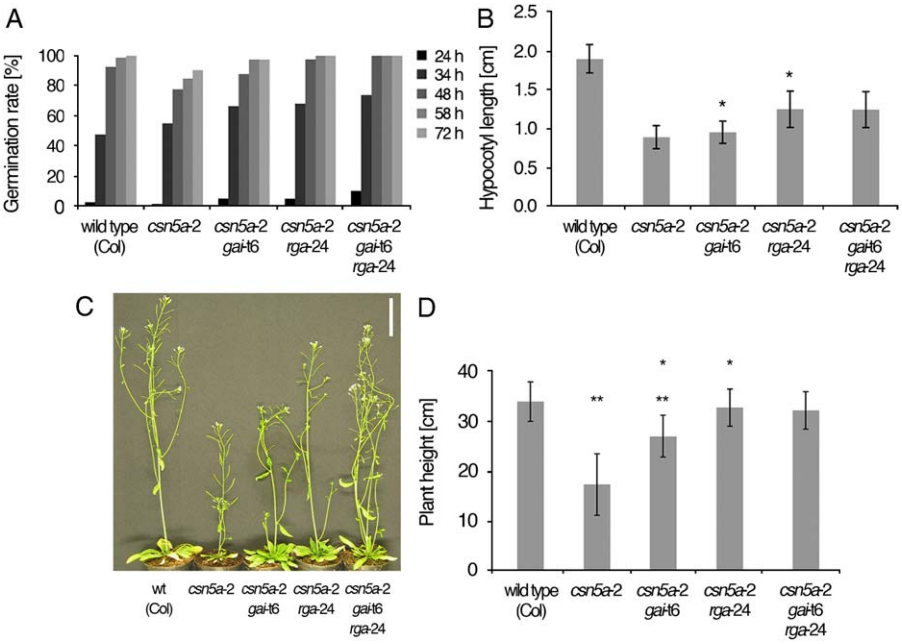

Fig. 4. RGA and GAI repress elongation in the *csn5a* mutants. (A) Germination rate of wild-type and *csn5a*-2 mutant seed as specified. $n \geq 30$. (B) Hypocotyl length of 7-day-old dark-grown wild type and *csn5a*-2 mutants. $n \geq 10$. The asterisk (∗) indicates a significant difference versus *csn5a*-2 mutants ($p < 0.005$; Student's t-test). (C) Phenotypes of 7-week-old wild-type and *csn5a*-2 mutant plants. Scale bar=5 cm. (D) Plant height of of 7-week-old wild-type and *csn5a*-2 mutant plants. The asterisks indicate significant differences between *csn5a*-2 and *csn5a*-2 *gai*-t6 (∗∗; $p < 0.005$; Student's t-test) or between *csn5a*-2 *gai*-t6 and *csn5a*-2 *rga-24* (∗; $p < 0.05$; Student's t-test).

be the major DELLA repressor in the GA-regulated control of germination (Lee et al., 2002)

CSN participates in the CRL-dependent protein degradation by removing the NEDD8 conjugation from the CRL cullin subunit. From mutant studies, it is understood that both neddylation and CSN-dependent deneddylation are required for CRL function. Our degradation studies of the DELLA protein RGA show that RGA is partially but not fully stabilized in strong *csn* mutants. This is in contrast to the complete stabilization of RGA in the SCF^{SLY1} mutant *sly1*. Although this observation shows that CSN contributes to GA-dependent protein degradation via deneddylation of the cullin subunit of SCF^{SLY1}, it shows at the same time that CSN is not essential for CRL-mediated protein degradation. We have recently provided circumstantial evidence that also auxin-induced SCF^{TIR1}-dependent protein degradation is only partially impaired in *Arabidopsis csn* mutants and suggested therefore that CSN-mediated deneddylation of cullins is not essential for CRL function. Based on our observations and biochemical studies published by others, it can be postulated that CSN and deneddylation are not essential for CRL-mediated protein degradation. Instead, it appears that neddylation and deneddylation stabilize and destabilize CRL complexes, respectively, and that the de novo assembly or stabilization of substrate-specific CRLs such as SCF^{SLY1} in the case of GA responses is impaired in a deneddylation-deficient *csn* mutant but that a basal level of functional SCF^{SLY1} is present independent of the cullin neddylation status.

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