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

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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.,

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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*)