The *Arabidopsis* COP9 signalosome is essential for G2 phase progression and genomic stability

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The COP9 signalosome (CSN) is required for the full activity of cullin-RING E3 ubiquitin ligases (CRLs) in eukaryotes. CSN exerts its function on CRLs by removing the ubiquitin-related NEDD8 conjugate from the cullin subunit of CRLs. CSN seems, thereby, to control CRL disassembly or CRL subunit stability. In *Arabidopsis thaliana*, loss of CSN function leads to constitutive photomorphogenic (cop) seedling development and a post-germination growth arrest. The underlying molecular cause of this growth arrest remains to be determined.

Here, we show that *Arabidopsis* csn mutants are delayed in G2 phase progression. This cell cycle arrest correlates with the induction of the DNA damage response pathway and is suggestive of the activation of a DNA damage checkpoint. In support of this hypothesis, we detected gene conversion events in csn mutants that are indicative of DNA double-strand breaks. DNA damage is also apparent in mutants of the NEDD8 conjugation pathway and in mutants of the E3 ligase subunits CULLIN4, COP1 and DET1, which share phenotypes with csn mutants. In summary, our data suggest that *Arabidopsis* csn mutants undergo DNA damage, which might be the cause of the delay in G2 cell cycle progression.

KEY WORDS: COP9 signalosome, Cell cycle, DNA damage

INTRODUCTION

The COP9 signalosome (CSN) was originally identified based on mutants with a constitutive photomorphogenic (cop) phenotype in *Arabidopsis thaliana* seedlings (Wei et al., 1994). CSN is an eight-subunit protein complex in *Arabidopsis* and functionally homologous complexes have been identified in yeasts and animals (Chamovitz et al., 1996; Seeger et al., 1998; Wei and Deng, 1998; Freilich et al., 1999; Mundt et al., 1999; Wei et al., 2002; Busch et al., 2003). CSN physically interacts with cullin-RING-type E3 ubiquitin ligases (CRLs) and regulates their activity, assembly and/or subunit stability through the removal of the ubiquitin-related NEDD8 protein from the CRL cullin subunit (deneddleylation) (Lyapina et al., 2001; Schwechheimer et al., 2001; He et al., 2005; Bornstein et al., 2006; Chew and Hagen, 2007; Cope and Deshaies, 2006).

Typically, the loss of one CSN subunit impairs the function of the entire protein complex, at least with regard to cullin deneddleylation. Whereas *csn* mutants from yeasts are viable, *csn* mutants from higher eukaryotes arrest growth during the early stages of development (Chamovitz et al., 1996; Freilich et al., 1999; Mundt et al., 1999; Mundt et al., 2002; Busch et al., 2003; Lykke-Andersen et al., 2003; Yan et al., 2003; Tomoda et al., 2004). All *Arabidopsis* CSN subunit mutants are phenotypically indistinguishable and arrest growth at the seedling stage (Kwok et al., 1996). The molecular cause of this growth arrest remains to be determined.

Since *csn* mutants are impaired in CRL function, they fail to efficiently degrade CRL substrates. The accumulation of these degradation targets is thought to be the molecular cause of the *csn* mutant phenotypes. In some cases, it has been possible to identify crucial degradation substrates that are responsible for *csn* mutant phenotypes. *Schizosaccharomyces pombe* *csn1* and *csn2* mutants (but, interestingly, not *csn4* and *csn5* mutants) are delayed in their progression through S phase (Mundt et al., 1999; Zhou et al., 2001; Mundt et al., 2002; Wei et al., 2002). In the wild type, the CRL-dependent degradation of the ribonucleotide reductase (RNR) repressor Spd1 is essential for full RNR activity and dNTP synthesis during S phase. The S phase delay of the *csn1* mutant is caused by Spd1 accumulation and can be suppressed by overexpression of RNR or by deletion of Spd1. These findings show that Spd1 is a crucial repressor in fission yeast *csn1* mutants (Liu et al., 2003; Bondar et al., 2004).

In fission yeast CSN mutants, cyclin E has been recognized as the crucial regulator for the G1–S transition during early oogenesis, and the genetic deletion of cyclin E suppresses CSN mutant phenotypes at this developmental stage (Doronkin et al., 2003). In mice, the deletion of *Csn2*, *Csn3* and *Csn5* (*Cops2*, *Cops3* and *Cops5*) causes lethality during embryogenesis (Lykke-Andersen et al., 2003; Yan et al., 2003; Tomoda et al., 2004). This growth arrest correlates with the accumulation of the G1 cell cycle regulators p53 (Trp53), p27Kip1 (Cdkn1b) and cyclin E, and ultimately induces apoptosis. However, a crucial growth repressor has not been identified in CSN mutant mice. In *CSN4* and *CSN5* (COPS4 and COPS5) siRNA human cell lines, the CRL subunit and F-box protein SPK2 is destabilized (Denti et al., 2006). The cell cycle progression defect of these siRNA lines can be suppressed by SKP2 overexpression. This result was interpreted as indicating that CSN is required for SKP2 stability and that the cell cycle arrest is induced by the accumulation of p27Kip1 (He et al., 2005; Cope and Deshaies, 2006; Denti et al., 2006).

The mutant screens that resulted in the identification of *csn* mutants also resulted in the identification of the constitutively photomorphogenic *cop1* and *det1* mutants from *Arabidopsis*.
COP1 is a RING-type E3 ubiquitin ligase that mediates the degradation of several positive photomorphogenesis regulators (Osterlund et al., 2000; Seo et al., 2003; Seo et al., 2004). The human COP1 ortholog (RFWD2) has been implicated in c-JUN degradation and in DNA damage response following irradiation (Dornan et al., 2004; Wertz et al., 2004). Human COP1 is inactivated in response to DNA damage by ATM-dependent phosphorylation, and the consequent stabilization of its degradation target p53 induces a G1 cell cycle arrest (Dornan et al., 2004). The function of DET1 only became clear when it was recognized that it is a subunit of a CULLIN4 (CUL4)-containing CRL, designated DCXDET1COP1 or CUL4-DDB1DETICOP1, which also includes COP1 and the adaptor subunit DAMAGED DNA-BINDING PROTEIN1 (DDB1) (Benvenuto et al., 2002; Dornan et al., 2004; Wertz et al., 2004; Yanagawa et al., 2004; Chen et al., 2006). Although these findings suggest that COP1 and DET1 function together in a CUL4-containing CRL, the COP1 monomer alone has in vitro E3 ligase activity. It is therefore presently unclear which functions of COP1 require the E3 complex (and DET1) and which functions are mediated by COP1 alone.

Arabidopsis csn mutants arrest growth at the seedling stage. The underlying molecular cause of this growth arrest remains to be identified. Here we show that csn mutant cells have a delay in G2 phase progression. This delay correlates with the activation of the DNA damage response pathway but is not exclusively induced by the DNA damage signaling kinases ATAXIA TELANGECTASIA MUTATED (ATM) or WEE1. Our observation that gene conversion events can occur in csn mutants strongly argues that DNA double-strand breaks are at least one type of DNA damage that is present in these mutants. We thus propose that DNA damage might be the cause of the csn mutant growth arrest.

MATERIALS AND METHODS

Biological material

All experiments were carried out with Arabidopsis thaliana ecotype Columbia. The following T-DNA insertion mutant alleles were obtained from the Nottingham Arabidopsis Stock Centre (NASC): csn3-1 (Salk_000593), csn4-1 (Salk_043720) and csn5a-1, csn5a-2 and csn5b-1 as well as the csn5a-2 csn5b-1 (csn5ab) double mutant (Dohmann et al., 2005; Rubio et al., 2005). The atm-2 (Salk_929606) mutant and the IU.GUS transgenic lines were made available by Holger Puchta (University of Karlsruhe, Germany) (Orel et al., 2003). Other previously reported mutant lines used were: wee1-2 (SALK_147968 (De Schutter et al., 2007)); cop1-1, cop1-5, cop1-6 (Deng and Quail, 1992); det1-1 (Chory et al., 1989); csn4-1 (GABI-Kat 600H03 (Bernhardt et al., 2006)); axr1-3, axr1-12 (LEYER et al., 1993); det2 (Schumacher et al., 1999).

The PARP2.GUS (De Schutter et al., 2007), WEE1:GUS (De Schutter et al., 2007), and CYCB1:1:GUS (Ferreira et al., 1994) transgenes were introgressed into the csn mutant backgrounds. For GUS staining, 7-day-old seedlings were fixed for 15 minutes in hexane and stained for 2 hours (CYCB1:1:GUS), 5 hours (PARP2:GUS), or 12 hours (WEE1:GUS and IU.GUS) with GUS-staining solution (100 mM Na-phosphate buffer pH 7.0, 0.5 mM K4Fe(CN)6, 0.5 mM K3Fe(CN)6, 0.1% Triton X-100, 0.5 mg/ml X-Gluc), and subsequently destained in 70% ethanol. CYCB1:1:GUS-stained seedlings were mounted on slides with chloralhydrate:H2O:glycerol 20:9:3 for microscopic analyses. CYCB1:1:GUS expression following a 24- or 48-hour treatment with the synthetic auxin 2,4-dichlorophenoxy acetic acid (2,4-D; 1 μM) was examined in the same manner. Photographs were taken with an Axioskop (Zeiss, Oberkochen, Germany). atm-2 and wee1-2 were crossed into the csn5-1, csn4-1 and csn5ab (atm only) mutant backgrounds. For genotyping, genomic DNA was prepared from the cotyledons of F2 progeny seedlings with a csn mutant phenotype using primers specific for the wee1-2 and the atm-2 mutations. Following genotyping, roots from 7-day-old seedlings with the single or double mutant genotype were pooled and analyzed by FACS with regard to their cell cycle distribution (Boudolf et al., 2004). For a list of primers and information for genotyping, see Table 1.

Microarray analysis

The csn mutant experiment was conducted with wild-type (Columbia) and csn3, csn4, as well as csn5ab mutant seedlings that had been grown for 7 days on solid growth medium in the dark or in the light. RNA for microarray analysis was extracted from three biological replicates for each sample using the RNeasy Kit (Qiagen, Hilden, Germany). Complementary RNA was prepared from 6 μg total RNA of each replicate as described in the Affymetrix Expression Analysis Technical Manual using the One-Cycle Target Labeling Protocol. Microarray labeled RNA was hybridized to Affymetrix ATH1 whole-genome arrays and scanned using the Affymetrix GeneChip Scanner 3000. The expression data were normalized using the GCRMA method (Wu et al., 2004) and filtered for probesets with a probe-level signal (PM) greater than the median of the background probesets. Gene expression values were calculated using the robust multiarray average method (Bryan et al., 1999). Microarray data were deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE17359.

Table 1. List of primers and information for genotyping and RT-PCR

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Genotyping combinations

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Primers for expression analysis

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<td>RAD51 RV</td>
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and Control Reagents (Affymetrix, Santa Clara, CA). In brief, double-stranded cDNA was synthesized and biotin-labeled target cRNA was prepared by cDNA in vitro transcription in the presence of biotinylated UTP and CTP. After purification, cRNA was fragmented and used to hybrizde the *Arabidopsis* ATH1 GeneChip array (Affymetrix). Hybridization, washing, staining, scanning, and data collection were performed for each replicate sample independently in an Affymetrix GeneChip Fluidics Station 450 and GeneArray Scanner. The raw microarray data were normalized (geRMA) and analyzed using the Genespring GX package (Agilent Technologies, Santa Clara, CA). Raw data were submitted to the Gene Expression Omnibus (GSE9728). The microarray data from light-grown wild-type and *csn4* mutant seedlings have been analyzed and published in a different context [GSM88049-GSM88091, GSM88055-GSM88057 (Schwager et al., 2007)].

For the expression analysis of the 57 core cell-cycle genes (see Table S1A in the supplementary material), the data were analyzed by one-way ANOVA (Benjamini and Hochberg false discovery rate <0.05) using default settings to control for false positives (Benjamini and Hochberg, 1995; Menges et al., 2005). The 44 genes resulting from this analysis were then filtered with regard to their differential expression (2-fold induction or repression) in the dark- and light-grown *csn* mutants compared with the respective wild-type samples (see Table S1C in the supplementary material). Eight genes were identified as being differentially expressed in the dark as well as in the light (see Table S1C in the supplementary material). A gene tree was generated from these genes using the default setting of the gene tree clustering algorithm, and visualized using the Genespring GX 7.3 software.

Based on a subset of publicly available microarray data (GSE5625; 0 hour, 1 hour, 3 hours, 6 hours and 12 hours of untreated and bleomycin- and mitomycin-treated *Arabidopsis* shoots), which were generated as part of the AtGenExpress Initiative (www.arabidopsis.org/info/expression/ATGenExpress.jsp), we identified 12 differentially expressed genes (one-way ANOVA using default settings, Benjamini and Hochberg false discovery rate <0.05; 2-fold induction or repression 3 hours and 6 hours after treatment with the DNA-damaging agents bleomycin and mitomycin; Genespring GX 7.3 software) from a list of 150 genes (www.uea.ac.uk/~h270/repair.htm) with a known or predicted function in DNA repair (see Table S2 in the supplementary material). A gene tree was generated from these genes using the default setting of the gene tree clustering algorithm and visualized using the Genespring GX 7.3 software.

RT-PCR of a small number of differentially expressed genes was used to confirm the microarray expression data (Frohmann et al., 1988). Total RNA was extracted from 90 mg 7-day-old light-grown seedlings using the Qiagen RNAeasy Kit and 3 µg RNA was used for reverse transcription with M-MuLV Reverse Transcriptase (Fermentas, St Leon-Rot, Germany) primed with the oligo-dT primer 5'-GACCTGAGTCGACATCGA(17T)-3'. One µl of the reverse transcription reaction was used for PCR with specific primers (Table 1) for the cell cycle markers *CYCA2;2* (AT5G13000) and *CYCB1;1* (AT4G37740), and the DNA damage markers BRC1 (AT4G21070), *PARP1* (AT4G02390) and *RAD51* (AT5G20850). *ACTIN2* (AT3G18780) served as a loading control.

**Immunoblots and immunostaining**

For immunoblots, total proteins were extracted from light-grown seedlings in protein extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, 1:100 Sigma Proteinase Inhibitor Cocktail; Sigma-Aldrich, Taufkirchen, Germany). Proteins (20 µg) were separated in a 10% SDS-PAGE gel and used for immunoblotting with antibodies against CDKB1;1 and KNOLLE (KN) (Lauber et al., 1997; Porceddu et al., 2001). Proteins for the western blot with anti-γH2AX (kindly provided by Anne Britt, Davis, CA) were extracted and detected as previously described (Friesner et al., 2005). For immunostaining with the anti-KN and anti-tubulin antibodies, 7-day-old *csn* mutant seedlings and 2-day-old (same size as 7-day-old *csn* mutants) or 5-day-old wild-type seedlings were examined as described previously (Lauber et al., 1997). The γH2AX immunostaining was performed in an identical manner.

**TUNEL assay**

For the TUNEL assay, 7-day-old dark-grown seedling roots were fixed and prepared in the same manner as for immunostaining. Following tissue permeabilization, the TUNEL reaction was performed using the In Situ Cell Death Detection Kit Fluorescein (Roche, Mannheim, Germany). For the positive control, wild-type roots were incubated for 10 minutes with 20U DNase I (Sigma-Aldrich) in 50 mM Tris-HCl pH 7.5, 1 mg/ml BSA, 1 mM MgSO4 and subsequently washed three times with PBS buffer prior to the TUNEL reaction. For the negative control, label solution lacking the enzyme was added. For DAPI staining, slides were incubated with 1 µg/ml 4',6-diamidine-2-phenylindole (DAPI) for 30 minutes, washed three times with PBS and embedded with antifade [PBS containing 90% glycerol and 25 mg/ml 1,4-diazabicyclo(2.2.2)octane, pH 9.5]. Fluorescence microscopy was performed with a Leica Confocal SP2 (Leica, Heidelberg, Germany).

**RESULTS**

Gene expression data suggest cell cycle defects in *csn* mutants

To understand the possible cause of the post-germination growth arrest of *Arabidopsis* *csn* mutants, we used Affymetrix ATH1 GeneChips to conduct gene expression analyses with 7-day-old dark- and light-grown *Arabidopsis* wild-type seedlings and *csn3-1* (designated hereafter *csn3*), *csn3-1* (*csn4*), and *csn3a-2 csn5b-1* (*csn5ab*) mutants (Fig. 1A) (Dohmann et al., 2005; Gusmaroli et al., 2004; Peng et al., 2001; Serino et al., 1999). These *csn* mutants are defective in the functions of CSN subunits CSN3, CSN4 and CSN5, respectively, and the analysis of these three mutants serves to show that the observations made here might hold true for all CSN subunit loss-of-function mutants. Amongst others, we analyzed the *csn* mutant microarray data with regard to the expression of a previously defined set of 57 cell cycle regulatory genes (Menges et al., 2005).

This analysis identified eight cell cycle regulatory genes that are...
differentially expressed (2-fold induction or repression) in all three csn mutants by comparison with the wild type, regardless of the growth condition (Fig. 1B and see Table S1 in the supplementary material). The putative CYCLIN-DEPENDENT KINASE (CDK) inhibitor KRP4 is the only gene from our analysis whose expression was reduced in csn mutants. The expression of the seven other genes was upregulated in csn mutants: CDK regulatory subunit CKS2, CDKB1;2, the protein kinase WEE1, the transcriptional regulator DEL3, as well as the cyclins CYCA2;2, CYCB1;1 and CYCA3;1. Remarkably, with the exception of WEE1, which is expressed during S phase or in response to DNA stress, all of these genes are differentially expressed during the cell cycle (Menges et al., 2005; De Schutter et al., 2007). Taken together, their expression in csn mutants can be interpreted as suggesting that csn mutants contain an increased number of cells in the G2 or M phase (Boudolf et al., 2004; Menges et al., 2005). Less stringent analyses of the microarray data resulted in the identification of a larger set of differentially expressed cell cycle regulatory genes (data not shown). Also, their expression pattern largely supports the notion that csn mutants contain an increased number of G2 or M phase cells. This was further substantiated by our finding that the expression of CYCA2;2 and CYCB1;1, the transcription of which is known to be strongly increased in G2 and M phase, was upregulated in csn mutants when examined by RT-PCR (see Fig. S1 in the supplementary material).

**Evidence for a G2 phase delay in csn mutants**

To define the cell cycle phase distribution in csn mutants at the cellular level, we crossed the CYCB1;1:GUS cell cycle reporter into the csn mutants. CYCB1;1:GUS confers the CYCB1;1 promoter-dependent expression of a fusion protein between the reporter protein β-glucuronidase (GUS) and the destruction box of the mitotic cyclin CYCB1;1 (Colon-Carmona et al., 1999). In wild-type cells, the CYCB1;1:GUS fusion protein is expressed in late S to mid-M phase cells, and it is degraded in cells that have passed mid-M phase. When compared with the wild type and in line with an increase in G2 phase cells, we detected an increased number of cells expressing the CYCB1;1:GUS reporter in cotyledons, the hypocotyl, the primary root meristem and in pericycle cells of csn mutant seedlings (Fig. 2A and data not shown). Next, we analyzed auxin-stimulated cell cycle activity in csn mutants. Auxin treatment of wild-type roots leads to the formation of lateral roots as a result of pericycle cell divisions. When we applied the synthetic auxin 2,4-dichlorophenoxy acetic acid (2,4D) to CYCB1;1:GUS-expressing wild-type plants, we observed the expected formation of lateral root primordia as well as strong CYCB1;1:GUS activity, which is indicative of increased cell division activity (Fig. 2A-C). By contrast, auxin-treated csn mutant seedlings had CYCB1;1:GUS-positive pericycle cells following auxin treatment, but their roots failed to produce lateral root primordia, indicating that these cells have passed G1 phase but are delayed in S, G2 or M phase (Fig. 2A-C). We further analyzed this apparent delay in cell cycle progression using immunoblot with antibodies directed against CDKB1;1 and KNOLLE (KN). CDKB1;1 is specific for the G2 and M phase and is required for the G2–M phase transition (Porceddu et al., 2001; Boudolf et al., 2004). KN is an M phase-specific syntaxin with a role in vesicle fusion during cytokinesis (Lauber et al., 1997). When we examined the accumulation of CDKB1;1 and KN in csn mutants, we detected increased levels of CDKB1;1 and reduced levels of KN as compared with the wild type (Fig. 2D). This finding thus supports the notion that csn mutants contain an increased number of G2 phase cells and a decreased number of cytokinetic cells.

**Cell divisions can occur in csn mutants**

To examine whether the altered expression of cell cycle markers is the result of a more general cell division or cytokinesis defect in csn mutants, we performed immunostaining with the anti-KN antibody. In wild-type cells, KN is detectable in vesicles that fuse with the newly formed cell plate only during M phase (see Fig. S2A-D in the supplementary material). In all three csn mutants, we detected apparently normal cytokinesis events, indicating that cell divisions can occur in csn mutant roots and that there are no abnormal cell divisions or cytokinesis defects (see Fig. S2E-J in the supplementary material). The observation that cell cycle activity is not fully impaired in csn mutants is also supported by our finding that,

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**Fig. 2. Cell cycle marker activity in csn mutants.** (A) CYCB1;1:GUS expression in primary roots (upper row) and root tips (lower row) of 7-day-old light-grown wild-type and csn3, csn4 and csn5ab Arabidopsis seedlings. (B,C) CYCB1;1:GUS expression in primary roots (upper row) and root tips (lower row) following 24 hours and 48 hours of treatment, respectively, with the synthetic auxin 2,4D. Arrowheads indicate lateral root primordia. (D) KN and CDKB1;1 accumulation in protein extracts (20 μg) of 2-day-old wild-type seedlings and 7-day-old csn3, csn4 and csn5ab mutant seedlings. A cross-reacting band was used as a loading control. Relevant bands are indicated by arrowheads. Wild-type and mutant plants of different ages were used for this analysis because 2-day-old wild-type and 7-day-old mutant seedlings are comparable in size and contain a comparable number of dividing cells. Scale bars: 1 mm in wild type; 0.5 mm in csn3, csn4 and csn5ab.
although inefficiently, cell divisions and callus formation can be induced from hypocotyls of csn mutants on callus induction media (E.M.N.D., unpublished). We thus conclude that cell divisions can occur in csn mutant roots and that G2 and/or M phase are not fully blocked, but rather delayed.

**Accumulation of G2 phase cells in csn mutants**

To assess the relative number of cells in the different cell cycle phases, we analyzed the DNA content of wild-type and csn mutant root cells by fluorescence-activated cell sorting (FACS) (Boudolf et al., 2004). The flow cytometric analysis indicated a strong accumulation of G2 phase cells with a 4C DNA content in all three csn mutants compared with the wild type (65-70% in the mutants versus 28-34% in the wild type; Fig. 3A-E and see Table S3 in the supplementary material). At the same time, hardly any endoreduplicated cells were detected in the csn mutants. These findings support the above-mentioned hypothesis that csn mutant cells delay division (and endoreduplication) after S phase. To rule out the possibility that the accumulation of G2 phase cells is a consequence (rather than cause) of the csn mutant growth arrest, we also examined the roots of viable mutant alleles of CSN5A and CSN5B, the two genes encoding CSN5 function in *Arabidopsis* (Dohmann et al., 2005; Gusmaroli et al., 2004). *csn5a* loss-of-function mutants are dwarfed but viable and fertile plants, whereas *csn5b* mutants are almost indistinguishable from the wild type and the contribution of CSN5B to plant growth and development is only evident in the *csn5ab* double mutant (Fig. 1A) (Dohmann et al., 2005). Interestingly, the FACS analysis revealed a correlation between the severity of the csn5 mutant phenotype and the increase in the number of cells with a 4C DNA content (*csn5a*-1 62%, *csn5a*-2 52% and *csn5b*-1 29.5% cells with 4C DNA content as compared with the wild type 28-34%) (Fig. 3F-H and see Table S3 in the supplementary material). Taken together, these results support the notion that the majority of csn mutant root cells are delayed in G2 phase, and that this defect is not a consequence of the growth arrest of csn-null mutants but more likely its cause.

**Activation of the DNA damage response pathway in csn mutants**

The observed accumulation of cells in the G2 phase suggests the activation of a cell cycle checkpoint in csn mutants. Since WEE1 transcription is induced upon the inhibition of DNA replication or following DNA damage, the WEE1 induction in csn mutants suggests that this is a consequence of DNA stress (Fig. 1B) (De Schutter et al., 2007). To assure genomic stability, eukaryotic cells contain a range of specialized proteins that sense DNA damage, mark the sites of damage and repair it (Bray and West, 2005; Sancar et al., 2004). ATM and ATM AND RAD3-RELATED (ATR) mark the sites of DNA strand breaks by phosphorylation of HISTONE H2AX (H2AX), and they activate the expression of specific downstream genes including, at least in plants, that of the cell cycle inhibitory WEE1 gene (Friesner et al., 2005; Shiloh, 2006; Culligan et al., 2006; De Schutter et al., 2007). If left unrepaired, DNA damage can lead to G2 cell cycle arrest; therefore, (unpaired) DNA damage might be responsible for the growth arrest observed in csn mutants.

In order to find marker genes for an activated DNA damage response pathway, we analyzed the response to DNA-damaging agents of 150 genes with a known or predicted function in DNA repair (www.uea.ac.uk/~h270/repair.htm) using an expression profiling experiment that was generated as part of the AtGenExpress Initiative (see Table S2A,B in the supplementary material; www.arabidopsis.org/info/expression/ATGen-Express.jsp). In this experiment, gene expression profiles were generated from wild-type shoots that had been treated with the DNA-damaging agents bleomycin and mitomycin. Our search identified 12 genes, including those encoding the *Arabidopsis* homologs of BRCA1, PARP, RAD17, RAD30 and RAD51, as being induced in response to DNA damage (Fig. 4A and see Table S2C in the supplementary material). Interestingly, the expression of these DNA damage marker genes was also induced in dark- as well as in light-grown csn mutants, thus confirming our hypothesis that the DNA damage response pathway is activated in these mutants (Fig. 4A and see Table S2C in the supplementary material). Further evidence for the induction of this pathway in csn mutants came from the analysis of a PARP2:GUS reporter construct (De Schutter et al., 2007). Whereas PARP2:GUS expression was low in wild-type cells but could be induced by the DNA-damaging agent bleomycin, PARP2:GUS was strongly expressed even in untreated csn mutant seedlings (Fig. 4B).

Since these observations suggested the presence of DNA damage in csn mutants, we tested whether other events associated with DNA damage could be detected. First, we examined H2AX phosphorylation, which is mediated by ATM and ATR and occurs at the sites of DNA strand breaks (Friesner et al., 2005; Shiloh, 2006). With an antibody specific for γH2AX, the phosphorylated form of H2AX, we detected increased levels of γH2AX in csn mutants as compared with the wild type (Fig. 5A). Second, we revealed the presence of specific γH2AX-positive subnuclear foci in csn mutants that are absent from wild-type cells and are reminiscent of the γH2AX nuclear foci that mark the sites of DNA damage, e.g. following ionizing radiation (Fig. 5B,C) (Rogakou et al., 1999; Friesner et al., 2005). Third, we showed that the reporter WEE1:GUS, which is induced in the wild type in response to DNA damage, is highly active in untreated csn mutants (Fig. 5D) (De...
So far, our results strongly support the notion that csn mutant nuclei contain free single or double DNA strands, which might be the result of DNA strand breaks or of problems during DNA replication or damage repair (Burma et al., 2001; Shilo, 2006). The repair of DNA strand breaks is essential for genomic stability and ultimately for the survival of all organisms (Sancar et al., 2004; Bray and West, 2005). DNA double-strand breaks (DSBs) are principally repaired by non-homologous end joining and by homologous recombination, the latter of which becomes the preferential DSB repair mechanism when homologous sequences are available nearby. In plants, the reporter construct IU.GUS enables examination of homologous recombination-mediated DSB repair and can thereby provide indirect evidence for the presence of DSBs (Fig. 6A) (Orel et al., 2003). We introduced the IU.GUS reporter into the csn mutants and examined the number of gene conversion events by counting the number of GUS-positive cells or sectors. GUS-positive cells were detected almost exclusively in csn mutant seedlings (Fig. 6B,C). Only a single GUS-positive sector was identified amongst 80 wild-type seedlings. The substantial increase in GUS-positive cells in the csn mutants indicates that DSBs occur in the reporter gene (and most likely also in many other genomic loci), and also that the protein machinery required for homologous recombination is functional in csn mutants (Fig. 6B,C).

ATM and WEE1 inactivation is not sufficient to overcome the G2 phase delay in csn mutants

The molecular phenotypes of csn mutants indicate activation of the ATM/ATR-dependent DNA damage checkpoint. WEE1 kinase, the transcription of which is induced in csn mutants, is an important downstream regulator of the ATM pathway in plants (Fig. 1B, Fig. 5D) (De Schutter et al., 2007). We reasoned that the activation of ATM or WEE1 kinases might be the cause of the G2 cell cycle delay in csn mutant cells. To test this, we introduced the atm-2 and wee1-2 loss-of-function alleles into csn mutant backgrounds, identified atm, wee1 and csn single and double mutants by genotyping, and examined their morphology as well as their cell cycle phase distribution using flow cytometry. These analyses showed that neither mutation alters the morphology or the cell cycle phase distribution phenotype of csn mutants (see Fig. S3 and Tables S4, S5 in the supplementary material). Taken together, these findings indicate that other signaling pathways mediate the cell cycle delay in csn mutants, possibly in cooperation with ATM and WEE1.

DNA damage in mutants of the neddylation pathway and the proposed CUL4-DDB1DETCOP1 E3 ligase

CSN regulates CRL assembly or subunit stability, and thereby ultimately CRL activity, through the removal of the ubiquitin-related NEDD8 protein from the cullin subunit of CRLs. Mutants deficient in CSN function accumulate neddylated cullins, including neddylated CUL4 (Lyapina et al., 2001; Schwechheimer et al., 2001; Dohmann et al., 2005). Arabidopsis mutants deficient in the NEDD8-activating enzyme regulatory subunit (axr1) have defects that are similar to those of the neddylation-deficient csn mutants (de Pozo et al., 2002; Schwechheimer et al., 2002). It has therefore been proposed that neddylation and deneddylation act together to control CRL assembly and function (de Pozo et al., 2002; Schwechheimer et al., 2002; Bornstein et al., 2006).
Arabidopsis cop1, det1 and csn mutants were identified in the same mutant screens based on their constitutive photomorphogenic phenotype (Kwok et al., 1996). Furthermore, there is evidence that CUL4, COP1 and DET1 function together in a CRL designated DCX\textsuperscript{COP1\textsubscript{DET1}} or CUL4-DDB1\textsuperscript{COP1\textsubscript{DET1}}, which is conserved in humans and in plants and requires CSN for full function (Wertz et al., 2004; Bernhardt et al., 2006). We therefore examined the roots of mutants deficient in the neddylation pathway and in CUL4-DDB1\textsuperscript{DET1\textsubscript{COP1}} function with regard to DNA damage and cell cycle phase distribution. Interestingly, the TUNEL assay indicated the presence of DNA single- or double-strand breaks in \textit{cul4} (\textit{cul4}-1; a weak \textit{cul4} mutant allele), \textit{cop1} (\textit{cop1}-4, \textit{cop1}-5 and \textit{cop1}-6) and \textit{det1} (\textit{det1}-1) mutants, as well as in \textit{axr1} (\textit{axr1}-3, \textit{axr1}-12) mutants, suggesting that DNA repair is deficient in these mutants (see Fig. S4 in the supplementary material; data not shown). By contrast, no DNA damage was apparent in roots of the \textit{det3} mutant, which was identified in the same mutant screen as \textit{det1} and is deficient in a subunit of the functionally unrelated vacuolar \textit{H}+\textsuperscript{+}-ATPase (see Fig. S4F in the supplementary material) (Schumacher et al., 1999). Flow cytometric analyses of the respective mutants indicated that the DNA damage observed in these mutants does not lead to alterations in the cell cycle phase distribution (see Fig. S3 and Table S3 in the supplementary material). This finding suggests that AXR1, CUL4, COP1 and DET1 are required for efficient DNA damage repair, but that the DNA damage can seemingly be repaired prior to cell division in these mutants (Kwok et al., 1996).

**DISCUSSION**

Previously published studies on the role of CSN in plant development have been concerned with its role as a regulator of CRLs from the light and phytohormone signaling pathways (Schwechheimer et al., 2001; Liu et al., 2002; Schwechheimer et al., 2002; Wang et al., 2003). This is the first report that reveals a crucial function for CSN in G2 phase cell cycle progression, in plants in particular and in eukaryotes in general. So far, csn mutant growth defects in mammalian systems have been correlated with the accumulation of the G1 cell cycle regulators p53, p2\textsuperscript{7Kip1} and cyclin E (Lykke-Andersen et al., 2003; Yan et al., 2003; Tomoda et al., 2004; Denti et al., 2006). The slow S phase progression of fission yeast \textit{csn} mutants has been explained by the stabilization of the RNR-repressor Spd1 (Bondar et al., 2004; Liu et al., 2003). Since the \textit{Arabidopsis} genome does not encode recognizable functional homologs of fission yeast Spd1 or of the mammalian G1 cell cycle regulators p53, p2\textsuperscript{7Kip1} and cyclin E, the accumulation of these

![Fig. 5. Evidence for DNA damage in *Arabidopsis* csn mutants.](image)

(A) Accumulation of the phosphorylated H2AX variant γH2AX in a histone preparation (13 μg) from 2-day-old (same size) and 7-day-old (same age) wild-type seedlings and 7-day-old \textit{csn3}, \textit{csn4} and \textit{csn5ab} mutant seedlings as detected with a γH2AX-specific antibody (Friesner et al., 2005). A Coomassie-stained band serves as a loading control. H2AX transcription was not altered between the wild type and the csn mutants (data not shown). (B, C) Immunostaining of wild-type and \textit{csn4} mutant root tip cells with the anti-γH2AX antibody (green) identifies subnuclear γH2AX-specific foci that may mark sites of damaged DNA in \textit{csn4} mutants (C, left panel). The samples were counterstained using the DNA stain DAPI. Scale bars: 5 μm. (D) Activation of the stress-induced WEE1:GUS reporter construct in wild-type seedlings, bleomycin-treated (12 hours, 5 μg/ml) wild-type seedlings and in untreated \textit{csn} mutants. Scale bars: 2 mm in wild type; 0.5 mm in \textit{csn2}, \textit{csn4} and \textit{csn5ab}. (E-M) Confocal images of primary roots of 7-day-old dark-grown wild-type and csn mutants following the TUNEL assay (left column). Roots were counterstained with DAPI (right column). For the positive control, fixed root material was subjected to treatment with DNase I. For the negative control, terminal transferase was omitted from the TUNEL reaction. The experiment was repeated three times and a representative image from one experiment is shown. Scale bars: 1 mm in wild type, \textit{csn5a} and \textit{csn5b}; 0.5 mm in \textit{csn3}, \textit{csn4} and \textit{csn5ab}. (N) Confocal images of a 7-day-old bleomycin-treated (12 hours, 5 μg/ml) dark-grown wild-type seedling following the TUNEL assay (left panel). Roots were counterstained with DAPI (right panel). Scale bar: 1 mm.
proteins cannot serve to explain the cell cycle defects observed in the *Arabidopsis csn* mutants. Therefore, the identity of the regulatory protein(s) that causes the G2 phase arrest in *Arabidopsis csn* mutants remains to be determined. Conversely, it can also be envisioned that CSN has an important role in G2 cell cycle progression in mammals.

Since we revealed the activation of a DNA damage checkpoint in *csn* mutants and because we showed that *csn* mutants repair DSBs, we propose that DSBs are at least in part responsible for the mutant growth arrest. Interestingly, *csn* mutants from *Aspergillus* are known to be hypersensitive to DNA-damaging agents. These mutants are, however, viable and their precise defect with regard to DNA damage repair is unknown (Lima et al., 2005). *Arabidopsis csn* mutants also display hallmark phenotypes that are indicative of activation of the ATM/ATR signaling pathway. Although our genetic analyses allow us to exclude the possibility that signaling by ATM or by WEE1 is responsible for the cell cycle defect in *Arabidopsis csn* mutants, we cannot and do not want to rule out that ATM and WEE1 contribute to the signaling events that lead to their growth arrest (see Fig. S3 in the supplementary material). In fact, there are already two reports that link ATM and CSN function. First, ATM was shown in a phosphoproteomics study to phosphorylate CSN subunits in response to DNA damage in animal cells (Matsuoka et al., 2007). Second, ATM was co-purified with CSN7 (and CSN) from plant extracts (Malec and Chamovitz, 2006). Although the biological relevance of these two findings is at present unclear, we consider ATM and its downstream effector WEE1 to be good candidates for regulators that induce the G2 cell cycle arrest in DNA-damaged *csn* mutants.

CSN regulates the assembly of CRLs or the stability of its subunits by deconjugation of NEDD8 protein from the cullins (Schwechheimer, 2004). The accumulation of as yet unknown CRL targets might be the molecular cause of the *csn* mutant growth arrest. Our experiments indicate that mutants of the neddylation pathway and mutants of the E3 ligase subunits CUL4, DET1 and COP1, have DNA damage. Since these mutants have normal cell cycle parameters and are viable, and because these mutants are (known to be) genetically stable, we conclude that their DNA damage can be efficiently repaired prior to cell division. Whereas *axr1* mutants, which are deficient in the function of one of two genes encoding the NEDD8-activating enzyme regulatory subunit in *Arabidopsis*, and *cul4* mutants (the *cul4-1* allele used in our study is a weak allele) are pleiotropic mutants in that they impair the function of many different CRLs, *cop1* and *det1* mutants are expected to specifically affect CUL4-DBD*DET1COP1* function (Schroeder et al., 2002; Wertz et al., 2004; Yanagawa et al., 2004; Bernhardt et al., 2006; Jin et al., 2006). To date, plant COP1 (and DET1) have primarily been investigated as proteins that promote the degradation of positive photomorphogenesis regulators in the dark (Osterlund et al., 2000; Seo et al., 2003; Seo et al., 2004). Based on our findings, CUL4-DBD*DET1COP1* can now also be implicated in DNA damage repair. Interestingly, human COP1 is important for p53 regulation in response to the ATM-executed DNA damage response and various other CUL4-containing E3 ligases have already been shown to play a role in DNA damage repair, such as CUL4-DBD*DET1COP1*, CUL4-DBD*DET1CSA* and CUL4-DBD*DET1CSA*COP1 (Groisman et al., 2003; Zhong et al., 2003; Hu et al., 2004; Kapetanaki et al., 2006; Bernhardt et al., 2006; Wang et al., 2006; Matsuoka et al., 2007; Sugimoto et al., 2008). Since *cop1* and *det1* mutants do not suffer from apparent cell cycle defects, our hypothesis is that CUL4-DBD*DET1COP1* acts redundantly with other E3 ligases, the function of which is not affected in the *cop1* and *det1* mutants.

Our experiment with the DNA repair reporter IU.GUS indicates that *csn* mutants contain DSBs, a finding that of course does not rule out the existence of other types of DNA damage in these mutants (Fig. 6). Nor does this experiment allow us to draw conclusions concerning the molecular cause of these DSBs. In the search for unstable proteins, the accumulation of which might explain these DSBs, our attention was drawn to a recent report that describes a crucial function of the DNA-licensing factor Cdt1 in maintaining genome stability (Tatsumi et al., 2006). Cdt1 is a degradation target of the above-mentioned CUL4-DBD*DET1* E3 ligase, and its degradation in human cells requires CSN (Higa et al., 2003). Cdt1 is normally degraded during S phase after the onset of DNA replication and its degradation is crucial to assure a single replication
round. However, Cdt1 also appears to be essential for maintaining genome stability during other cell cycle phases because its overexpression can induce DSBs and lead to severe chromosomal instability in quiescent cells (Tatsumi et al., 2006). Since Cdt1 and Cdt2 are conserved in plants, the deregulation of CUL4-DDB1\textsuperscript{-/-}DDB2 and the accumulation of Cdt1 could potentially be responsible for the DSBs in Arabidopsis csm mutants (Castellano Mdel et al., 2004; Kim and Kipreos, 2007).

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Supplementary material

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