The Arabidopsis Cop9 signalosome subunit 4 (CSN4) is involved in adventitious root formation

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The COP9 signalosome (CSN) is an evolutionary conserved multiprotein complex that regulates many aspects of plant development by controlling the activity of CULLIN-RING E3 ubiquitin ligases (CRLs). CRLs ubiquitinate and target for proteasomal degradation a vast number of specific substrate proteins involved in many developmental and physiological processes, including light and hormone signaling and cell division. As a consequence of CSN pleiotropic function, complete loss of CSN activity results in seedling lethality. Therefore, a detailed analysis of CSN physiological functions in adult Arabidopsis plants has been hampered by the early seedling lethality of csn null mutants. Here we report the identification and characterization of a viable allele of the Arabidopsis COP9 signalosome subunit 4 (CSN4). The allele, designated csn4-2035, suppresses the adventitious root (AR) phenotype of the Arabidopsis superroot2-1 mutant, potentially by altering its auxin signaling. Furthermore, we show that although the csn4-2035 mutation affects primary and lateral root (LR) formation in the 2035 suppressor mutant, CSN4 and other subunits of the COP9 complex seem to differentially control AR and LR development.

The CSN was first discovered in Arabidopsis, during a screening for mutants exhibiting constitutive photomorphogenic development in darkness, and was subsequently shown to be evolutionary conserved across eukaryotes (reviewed in ref. 1). The complex is composed of eight subunits, CSN1-CSN8. Six (CSN1-CSN4, CSN7 and CSN8) contain a PCI (Proteasome, COP9 signalosome and eukaryotic initiation factor 3, eIF3) domain, and two (CSN5 and CSN6) contain a MPN (Mrp1p-Pad1p-N-terminal) domain. In Arabidopsis, the PCI domain-containing subunits are encoded by single copy genes, while the MPN domain-containing subunits are each encoded by two highly homologous genes. The two genes encoding CSN5 (CSN5A and CSN5B) play unequal roles in the regulation of plant development, while CSN6A and CSN6B, the genes encoding the CSN6 subunit, act largely redundantly. The PCI and MPN subunits are structurally interdependent during the formation of the COP9 complex, thus explaining why loss of any of the eight CSN subunits leads to an apparently identical seedling lethal phenotype in Arabidopsis. Therefore, a detailed analysis of CSN physiological functions in adult Arabidopsis plants has been hampered by the early seedling lethality of the null csn mutants.

Before the availability of T-DNA insertion lines, the only known Arabidopsis csn mutants were the pleiotropic seedling lethal mutants, now collectively known as the cop (constitutively photomorphogenic)/det (de-etiolated)/fus (fusca), which were identified through genetic screenings (reviewed in refs 1 and 4). Recent identification of alleles with partial loss of CSN function has shed light on some aspects of CSN functions beyond the seedling stage. However, to our knowledge, viable csn mutants are available only for five of the eight CSN subunits, including the double encoded MPN domain-containing subunits CSN5 and CSN6, and only for three out of the six single copy gene-encoded PCI domain-containing subunits, CSN1, CSN2, and CSN3. It has been suggested that one potential reason for the lack of viable known csn mutants is that CSN-independent functions of CSN subunits can only be uncovered under specific conditions, and probably in particular types of screening. In this
More recently, the crystal structure of the human CSN6 for de-neddylation14–16. Therefore, the mutation of the Ala 302 could induce a destabilization of the CSN and/or the neddylated Cullin–RINGE3 ubiquitin ligases to CSN, which is subsequently communicated to CSN5 and COP9 signalosome has highlighted the important role of the PCI domain CSN4 subunit in sensing the binding substitution (Fig. 1a,b). The Ala 302 is part of a putative helix-loop-helix domain centered around amino acids 286–345, which is shown in a comparison of a fragment from the Arabidopsis CSN4 protein with homologs from other organisms. The position of highly conserved Ala302, mutated in csn4-2035, is highlighted. The position and nature of the amino acid substitution found in CSN4-2035 mutant protein is shown below, an alignment of CSN4 proteins from Arabidopsis thaliana (NP_199111.1; residues 286–345), Populus trichocarpa (XP_002320585.1; residues 286–345), Brachypodium distachyon (XP_003558584.1; residues 289–348), Oriza sativa (NP_001049272.1; residues 289–348), Drosophila melanogaster (NP_477444.1; residues 294–353) and Homo sapiens (NP_057213.2; residues 287–346), around Arabidopsis Ala 302 is shown.

Results and Discussion
Isolation of the 2035 mutant and identification of the csn4-2035 mutation. Aiming to identify new Arabidopsis genes involved in the control of adventitious root (AR) formation, we screened for suppressors of the superroot-1 (sur2-1) mutant10. We isolated, mapped and characterized a number of suppressors, and, for a subset of mutants, we identified the causal mutations in the corresponding genes9. Using the combined advantages of classical map-based-Cloning11 and whole genome re-sequencing12, we identified a point mutation in the locus At5g42970, which encodes the subunit 4 of the COP9 signalosome complex, as the potential causal mutation for the phenotype of one of the sur2-1 suppressors, designated 2035. The mutant carries a G-to-A mutation at position 2922 in the tenth exon of the CSN4 subunit gene, with the alleles used in this study.

Figure 1. The csn4 alleles used in this study. (a) Structure of the Arabidopsis CSN4 subunit gene, with the position of the csn4-2035 point mutation and of the two T-DNA insertion lines. Exons are indicated by black boxes, introns by lines. (b) A comparison of a fragment from the Arabidopsis CSN4 protein with homologs from other organisms. The position of highly conserved Ala302, mutated in csn4-2035, is highlighted. The position and nature of the amino acid substitution found in CSN4-2035 mutant protein is shown. Below, an alignment of CSN4 proteins from Arabidopsis thaliana (NP_199111.1; residues 286–345), Populus trichocarpa (XP_002320585.1; residues 286–345), Brachypodium distachyon (XP_003558584.1; residues 289–348), Oriza sativa (NP_001049272.1; residues 289–348), Drosophila melanogaster (NP_477444.1; residues 294–353) and Homo sapiens (NP_057213.2; residues 287–346), around Arabidopsis Ala302 is shown.

report, we introduce a viable allele of Arabidopsis CSN4, identified in a screening for mutants suppressing the adventitious root formation of the auxin overproducer superroot29.

Segregation analysis of the F2 progeny from a sur2-1gl1 × 2035 cross showed a 3:1 ratio of superroot:suppressor phenotype consistent with a single recessive mutation9. Therefore, the mutation of the Ala302 could induce a destabilization of the CSN and/or affect the de-neddylation process.
Figure 2. Phenotype and characterization of the *csn4* alleles. (a) The phenotype of *in vitro* grown 2035 suppressor mutant, together with Ws-4, Col-0, *sur2-1gl1, csn4-2035*, the two trans-heterozygotes double mutants, *sur2-1csn4-1/csn4-2035* and *sur2-1csn4-2/csn4-2035*, respectively, is shown, as compared to the non-viable *csn4-1* and *csn4-2* alleles. Seedlings were first etiolated in the dark, until their hypocotyls were 6 mm long, and then transferred to light for seven days to induce AR formation on the etiolated hypocotyls. Arrowheads indicate the root-hypocotyl junction; arrows indicate ARs. Bar, 5 mm. (b) Allelism test. A cross between the homozygous 2035 and the heterozygote *csn4-1* gives a 1:1 wild-type to mutant phenotype segregation ratio in the F1 generation. Arrowheads indicate the root-hypocotyl junction; arrows indicate ARs. Bar, 5 mm. (c) Numbers of AR were counted on the hypocotyls treated as in (a), and averaged. (d) Numbers of emergent lateral roots were counted on seedlings grown *in vitro* directly in light conditions for 10 d and averaged. (e) Primary root length was measured on the same seedling as in (d) and averaged. (f) Lateral root density was estimated...
Phenotypic characterization of the suppressor 2035. When grown in vitro on vertical plates, sur2-1gl1 developed numerous AR on the etiolated hypocotyls. The suppressor 2035, which carries the mutation csn4-2035 in a sur2-1gl1 background, developed significantly fewer AR compared to sur2-1gl1 (Fig. 2a,c). In addition, the AR formation was almost completely abolished in the csn4-2035 single mutant compared to the Ws-4 wild-type, since the majority of the seedlings had no initiated AR on the hypocotyl seven days after being transferred to the light, and only a limited number of individuals developed one AR (Fig. 2a,c). We had previously shown that the main root system of suppressor 2035 was also significantly reduced compared to sur2-1gl1 (Fig. 2d,e and ref. 9). Here we show that the main root system is also reduced in the csn4-2035 single mutant as compared to the Ws-4 wild-type. Both the primary root (PR) length and the lateral root (LR) number were significantly reduced (Fig. 2d,e,f), confirming that the csn4-2035 mutation also affects PR and LR formation.

Interestingly, the AR number vs LR number and PR length were differentially affected by the csn4-1/2035 and csn4-2/2035 heteroallelic combinations in the two trans-heterozygote double mutants, sur2-1csn4-1/2035 and sur2-1csn4-2/2035. While the average number of AR in the trans-heterozygote double mutants, sur2-1csn4-1/2035 and sur2-1csn4-2/2035 was not affected compared to 2035 (Fig. 2c), the PR length and the LR number and density were significantly decreased (Fig. 2d,e,f). As shown in Fig. 2c, the number of AR was not significantly different in the two populations derived from sur2-1csn4-1/2035 and sur2-1csn4-2/2035 compared to the 2035 suppressor. This was surprising, since it has been reported that the phenotype of a hypomorph allele had a more severe effect in trans to a deletion allele than when homozygous. Indeed, a more extreme phenotype in the populations containing 50% trans mutants was observed for PR and LR development, since the LR density was decreased in the two populations to 32% and 34%, respectively as compared to 2035 (Fig. 2d,e,f). It should be noted that in Fig. 2c-f, the data corresponding to the trans-heterozygotes sur2-1csn4-1/2035 and sur2-1csn4-2/2035 were collected from segregating populations including viable sur2-1csn4-1/2035 (50%) and 2035 (25%), and sur2-1csn4-2/2035 (50%) and 2035 (25%). These were generated by self-pollinating the sur2-1csn4-1/2035 and sur2-1csn4-2/2035 parents, respectively. In both situations, the segregating sur2-1csn4-1−/− (25%) and sur2-1csn4-2−/− (25%) had the characteristic fusc a phenotype and were not considered for the characterization. Apparently, losing one copy of the csn4-2035 mutation in 50% of the individuals from both populations did not have an impact on the AR number compared to the suppressor 2035, but this lack could not be compensated for in the case of PR and LR formation. This suggests that CSN4 and/or the COP9 complex differentially control AR and LR development.

When grown in soil, in LD conditions, both 2035 and csn4-2035 develop smaller rosettes than the wild type (Fig. 2g), and the adult plants show a mild dwarfism and loss of apical dominance phenotype as observed for other hypomorphic csn mutants.

The seedling and rosette phenotype of 2035 (short petioles, round leaves) is the result of the suppression of the sur2-1 phenotype. The apical part (not only the roots) loses the auxin related phenotype. Since the sur2-1 mutant phenotype is the result of auxin overproduction, its suppression may be due to either altered auxin homeostasis or auxin perception in the suppressor mutant. It has been reported that the viable CSN alleles csn1-10, csn2-5, csn3-3, csn5a and csn5b have altered auxin perception and auxin-resistant root growth. To test whether the csn4-2035 mutation suppresses the sur2-1 phenotype by disturbing the auxin signaling or the auxin homeostasis in the 2035 mutant, we measured the free IAA content both in the apical parts and the roots of in vitro grown seedlings. As we previously described in ref. 9 and show here in Fig. 2h, the 2035 mutant retains the high auxin level of sur2-1gl1, which indicates that the reduction of the AR number in 2035 is unlikely to be due to a reduced auxin content, suggesting a possible alteration in auxin signaling.

Auxin signaling is perturbed in 2035. It is well-known that the CSN plays a role in auxin signaling, acting as a de-neddylase to regulate SCFTIR1/AFB activity, and mutants altered in any CSN subunit are more or less resistant to inhibition of root growth by exogenously applied auxin and have reduced lateral root formation. Therefore, we performed a dose-response assay and measured the auxin inhibition of root elongation and found that csn4-2035 was resistant to 2,4-D compared to Ws-4 (Fig. 2i and Supplementary Fig. S1). After transfer to the medium supplemented with 0.1 μM 2,4-D, the wild type Ws-4 displayed inhibition of root elongation by 65% whereas csn4-2035 seedlings displayed a 20% inhibition only (Fig. 2i) and did not develop LR (Supplementary Fig. S1).
In previous work, we have demonstrated that a regulatory module, composed of three AUXIN RESPONSE FACTOR genes (ARF6, ARF8, ARF17, GH3.3, GH3.5 and GH3.6) controls AR initiation in Arabidopsis hypocotyls by modulating JA homeostasis. In addition, we recently showed that selected suppressor mutations of the sur2-1 phenotype differentially affected the expression of the ARF/GH3 regulatory module. In the suppressors analyzed, the reduced number of AR was independent of the endogenous auxin content, but positively correlated with the transcript amount of the three GH3 genes. In the present work we show that despite the endogenous content of free IAA in the suppressor 2035, which is still as high as that of the sur2-1gl1 mutant (Fig. 2h and ref. 9), the relative transcript amount of the GH3 genes is significantly reduced compared to sur2-1gl1 (Fig. 3a), probably explaining the reduced number of AR. Interestingly, this could be explained neither by a down-regulation of the expression of the positive regulators ARF6 or/and ARF8 nor an up-regulation of the negative regulator ARF17 (Fig. 3a). ARF transcriptional activity is negatively regulated by transcriptional repressors of the Aux/IAA family, which are unstable proteins rapidly degraded through the SCFTIR1/AFB1-dependent ubiquitin-proteasome system in the presence of a high auxin concentration. It was also shown that mutants altered in the expression of CSN3, CSN4, CSN5 or CSN8 subunits were partially impaired in the SCFTIR1/AFB1-dependent protein degradation, resulting in a partially altered auxin response. 

Figure 3. Auxin signaling is perturbed in 2035. (a) Quantification by quantitative real-time PCR of ARF6, ARF8, ARF17, GH3.3, GH3.5 and GH3.6 transcript abundance in hypocots of sur2-1gl1 and 2035 seedlings, which were etiolated until their hypocotyl had reached 6 mm (T0) and then transferred to the light for 72 h (T0+72 h light), or kept in dark for an additional 72 h (T0+72 h dark). Gene expression values are relative to the expression in the wild type, for which the value is set to 1. Error bars indicate standard error obtained from three independent biological replicates; (*) indicates values that were significantly different from sur2-1gl1 values according to one-way ANOVA combined with Bonferroni's comparison post-test; (ns) indicates values not significantly different; (P < 0.05; n = 3). (b) csn4-2035 is a weak allele mutant that produces as much protein as the wild type. CSN4 western blot analysis of protein extracts prepared from WS-4, csn4-2035 and the null allele csn4-1. (c) csn4-2035 mutation affects the de-neddylation activity of CSN4 protein. CUL1 western blot analysis of protein extracts prepared from WS-4 and csn mutant seedlings. The upper band indicates the modified (neddylated) CUL1. Ponceau stained polyvinylidene difluoride (PVDF) membrane is shown as a loading control. (d) Quantification by quantitative real-time PCR of GH3.3, GH3.5 and GH3.6 transcript abundance in hypocots of csn4-2035 seedlings. Gene expression values are relative to the expression in the wild type, for which the value is set to 1. Error bars indicate standard error obtained from three independent replicates; (*) indicate values that were significantly different from wild-type values (P < 0.05; n = 3).
Figure 4. The csn4-2035 mutation affects red and blue light perception in a sur2-1gl1 background. All measurements were performed on three independent biological replicates with a minimum of 40 seedlings each, as described in the Methods. Error bars indicate standard error. One-way ANOVA combined with Tukey’s multiple-comparison post-test revealed that the values indicated by (*) were significantly different from Ws-4 values; (▲) indicates values significantly different from sur2-1gl1 values; while (○) and (□) indicate values not significantly different from Ws-4 and sur2-1gl1 values, respectively; (P < 0.05; n > 120).

Because the CSN cleaves the RUB/NEDD8 peptide from the cullin subunit of CRL ubiquitin-ligases, we checked whether or not the csn4-2035 mutation affected CUL1 modification. We first showed that the csn4-2035 mutant produced as much CSN4 protein as the wild type, while the null allele mutant retained no protein as expected (Fig. 3b). These results suggested that the phenotype was not the result of a reduced amount of CSN4 protein but most likely due to a modification of its activity or to a defect in the CSN assembly or structure. Therefore, we analyzed de-neddylation activity (Fig. 3c). We included csn1-10 and csn3-3 mutants as controls because they were shown to exhibit similar auxin resistance but had different de-neddylation activity6. Figure 3c shows that there was no de-neddylation of CUL1-NEDD8 in the csn4-1 null allele mutant as expected and a clear accumulation of de-neddylated CUL1 in the wild type and csn3-3 mutant as reported in previous studies6. In contrast, we observed an accumulation of CUL1-NEDD8 in csn4-2035, as is the case in csn10-1, indicating that the csn4-2035 mutation partially affects the de-neddylation activity of the CSN and, as a consequence, decreases auxin sensitivity. This hypothesis is supported by the slight but significant down-regulation of the auxin inducible GH3 genes in the csn4-2035 mutant (Fig. 3d).

Therefore, the down-regulation of the auxin inducible GH3 genes in the 2035 suppressor mutant, resulting in the reduced number of AR, can reasonably be explained by an inefficient degradation of AUX/IAA proteins and, as a consequence, the down-regulation of the auxin signaling pathway which in turn would induce the expression of the activating ARFs such as ARF6 and ARF8 in a regulatory feedback loop. This would explain why, despite high endogenous auxin content and a combined increase in the relative transcript amount of the ARF6 and ARF8 positive regulators and decrease in the transcript amount of the ARF17 negative regulator, compared to sur2-1gl1 (Fig. 3a), the mutant 2035 develops fewer AR.

csn4-2035 mutation affects blue and red light perception in a sur2-1 background. No obvious csn phenotypes - cop/det (open cotyledons, short hypocotyl and absence of apical hook of the dark-grown seedling) and fuscus (accumulation of anthocyanins) - typical of the other reported Arabidopsis mutants of all six PCI-domain subunits, including the two CSN4 T-DNA alleles csn4-1 and csn4-2, were observed, either in 2035 mutant or in the csn4-2035 single mutant (Fig. 2a). Grown in vitro, in LD conditions, the hypocotyl of 2035 had an intermediate length between that of the wild-types and sur2-1gl19. Early observations have shown that light-grown CSN null mutants have very short hypocotyls23. Shorter hypocotyls than the wild-type controls have also been observed in week CSN alleles18,19, and the role of CSN in hypocotyl elongation has recently been demonstrated24. In a screening for novel CSN interactors, the authors identified CFK1 (COP9 INTERACTING F-BOX KELCH 1), a new plant-specific CSN-interacting F-box protein that is regulated by the CSN and the proteasome-dependent proteolysis. This physical interaction suggests that CFK1 function might be required for CSN-mediated hypocotyl inhibition, as demonstrated by enhanced hypocotyl length in the double mutant csn5a-2 CFKRNAi compared to either parents25. The authors have shown that light induces accumulation of the CFK1 transcript in the hypocotyl, and that CFK1 promotes hypocotyl elongation by increasing cell size. Moreover, while reduction of CSN levels enhances the short hypocotyl phenotype of CFK1RNAi seedlings, complete loss of CSN activity suppresses the long-hypocotyl phenotype of CFK1-overexpressing seedlings. In light of these findings, we can speculate that suppression of the hypocotyl growth in the 2035 suppressor mutant compared to sur2-1gl1 could be explained by a reduced activity of the CSN due to the mutation in the CSN4 subunit.
In Arabidopsis, normal hypocotyl elongation is controlled both by cryptochromes, which respond to blue/UV-A light, and phytochromes, which sense red/far-red light. An allelic mutant of sur2-1, red1 (redetiolated1), has been identified during a screening for mutants that display enhanced etiolation in continuous red light (cR), suggesting a defect in red light perception25. In addition, the CSN was originally discovered as an essential complex that regulates light-induced development in Arabidopsis26–29, and increased photomorphogenic responses were observed in different light conditions for viable alleles of the CSN subunit genes, csn5a, csn5b and csn6a-1 and csn6a-2, respectively30. To investigate further whether the csn4-2035 mutation alters light perception, we measured the hypocotyl length of 2035, sur2-1gl1 and Ws-4 under five fluence rates of continuous red (cR) and continuous blue (cB) light. In the dark, 2035 and sur2-1gl1 had the same hypocotyl length and were shorter than the wild type, as previously described for sur2-1 (Fig. 4a,b and ref. 10). When grown under cR at 40 or 210 μmol m\(^{-2}\)s\(^{-1}\) irradiance, both sur2-1gl1 and 2035 were longer than the wild type. Nevertheless the 2035 mutant had a significantly longer hypocotyl compared to sur2-1gl1 when grown under 1, 3 or 11 μmol m\(^{-2}\)s\(^{-1}\) irradiance (Fig. 4a), suggesting that a mutation in the CSN4 subunit affects red light perception. Similarly, a significant difference between the suppressor mutant 2035 and sur2-1gl1 was observed when seedlings were grown under cB light at 2 and 10 μmol m\(^{-2}\)s\(^{-1}\) irradiance (Fig. 4b). From these data, we can conclude that a mutation in the CSN4 subunit affects red and blue light perception to some extent, but we cannot say whether the AR phenotype and the light related phenotype are connected, and additional experiments are required. Nevertheless, we have previously shown that AR initiation is also controlled by light26,31, as well as by a signaling cross-talk between auxin and jasmonate involving MYC2 transcription factor32, therefore the csn4-2035 mutation could also highlight cross-talk between light and these signaling pathways in the control of AR initiation. Indeed, it was recently suggested that inhibition of hypocotyl elongation by jasmonates is enhanced under red light in phyB-dependent manner33, and that JA, MYC3, and transcription factors MYC2 and MYC4 short-lived proteins degraded by the proteasome, and stabilized by JA and light, in Arabidopsis thaliana. Darkness and CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) destabilize MYC2, MYC3, and MYC4 proteins, whereas red and blue lights stabilize them through the activation of the corresponding photoreceptors32. Therefore, the csn4-2035 mutation, by perturbing red and blue light perception, may also affect the stabilization of MYC proteins and thereby have an impact on adventitious root formation.

CSN subunits play differential roles in AR and LR formation. The CSN regulates multiple plant hormone signaling and developmental processes through SCF-type CRLs, known as CSF complexes, which act in many pathways such as SCF\(_{TIR1}\) in auxin7,20, SCF\(_{COI1}\) in jasmonate23,34 and SCF\(_{LYS}\) in gibberellic acid signaling35, SCF\(_{FDC}\) in flower development36, and SCF\(_{CFK1}\) in hypocotyl elongation24. The CSN also controls photomorphogenic development and abscisic acid signaling through a CUL4-CDD (COP10, DDB1 and DET1)37, and a CRL4-DDA1 complex38, respectively. The isolation of viable csn mutants provides an opportunity to study CSN functions beyond seedling stages by elucidating the role CSN and its individual subunits play in these processes. Interestingly, the viable CSN mutant alleles described so far, both for the double encoded MPN subunits and single encoded PCI subunits, either display pleiotropic developmental defects or have subtler phenotypes. The first viable csn mutants described were the loss-of-function T-DNA insertion mutants in the double encoded MPN domain-containing subunit CSN5A and CSN5B, csn5a-1 (null), csn5a-2 (hypo/morphic), and csn5b-139. The csn5a mutant allele displays increased photomorphogenic responses and pleiotropic developmental defects both at the seedling and adult stage, affecting lateral root and root hair formation, and flower size. In contrast, the csn5b mutant shows comparatively subtle phenotypes40. However, both csn5a-1csn5-b and csn5a-2csn5b double mutants mimic the phenotype of the previously described cop/det/fus mutants, indicating that the two CSN5 genes have redundant functions, with CSN5A having a stronger relative contribution to the respective phenotype than CSN5B. On the other hand, except for a very mild photomorphogenic phenotype in the dark and under blue light, the T-DNA insertion mutants in the CSN6A and CSN6B genes, coding for the second MPN subunits41, do not display any obvious morphological defects in white light and after the seedling stage, while loss of function for both CSN6 proteins leads to seedling lethality and a fusca phenotype in the double mutant csn6a-1csn6b-139.

Of the three single gene encoded PCI subunits for which viable alleles have been identified, csn1-10 has been described to have severe pleiotropic developmental defects associated with altered auxin responses42, while csn2-5, except for a mild dwarfsim of adult plants43 and a “curly hypocotyl” phenotype when seedlings were grown in darkness44 and csn3-345, although impaired in auxin responses, retain a wild-type-like phenotype, under the conditions investigated and for the analyzed parameters.

In order to test whether the viable csn mutants show differences in AR formation and whether they differentially interact with sur2-1, we counted the AR number in the single mutants csn1-10, csn2-5, csn3-3, csn5a-1, csn5a-2, and csn5b and in the double mutants with sur2-1. As shown in Fig. 5a, the AR number varied considerably among the tested csn mutants. The csn1-10 and csn3-3 mutants developed significantly fewer AR as compared to Col-0, and csn5a-1 and csn2-2 did not form any AR, while csn2-5 was not significantly different from its corresponding wild-type Ler (Fig. 5a). Interestingly, in contrast to csn5a-1 and csn2-2, the csn1-10 mutant developed an increased number of AR compared to the wild-type Col-0 (Fig. 5a), and although all csn mutants suppress the AR phenotype of sur2-1 to various degrees, csn5b enhanced the sur2-1 phenotype (Fig. 5b). This indicates that the two CSN5 subunits differentially contribute to the regulation of AR formation.

As shown in Fig. 2c,d, the csn4-2035 mutation differentially impacted on AR vs. LR development. We wondered whether this was also true for mutations in other CSN subunits. We counted the average length of LR on the seedlings of the csn1-10, csn2-5, csn3-3, csn5a-1, csn5a-2 and csn5b single mutants, and in the double mutants with sur2-1 grown for 10 days under light (Fig. 5c,d). Interestingly, all single and double mutants (except sur2-1csn5b double mutant) showed a reduced number of LR compared to the wild-type controls (Fig. 5c) or sur2-1 (Fig. 5d). Interestingly the number of LR in the sur2-1csn5b double mutant was not significantly different from
Analyzing the impact that the individual csn mutations have on AR vs. LR development, we can see that the number of both AR and LR is significantly reduced in csn1-10, csn3-3, csn5a-1, csn5a-2 single mutants compared to Col-0, as well as in their double mutants with sur2-1, compared to sur2-1gl1. In contrast, the csn2-5 mutant is affected with respect to LR number, but shows no significant difference in AR number compared to the Ler wild type, although the csn2-5 mutation strongly suppresses the sur2-1 phenotype in the double mutant sur2-1csn2-5 (Fig. 5a). The csn5b mutant, although showing a significant reduction of the number of LR, developed significantly more AR on the hypocotyl compared to Col-0. Moreover, it enhanced the AR phenotype in the double mutant sur2-1csn5b, while the LR number was not significantly different from sur2-1gl1.

Mutations in CSN subunits have previously been shown to impact LR formation in the viable identified single csn mutants or generated double mutants. While LR numbers in the single mutants csn1-10 and csn3-3 were comparable to wild-type control, single csn5a-2, csn5b-1, as well as double mutants csn3-3 csn1-10, developed significantly fewer LR. This reduction was attributed to the impaired auxin response of the mutants\(^a\), or to a defect in sur2-1gl1.

**Figure 5.** CSN subunits play differential roles in AR and LR formation. (a,b) Adventitious roots were counted on seedlings first etiolated in the dark, until their hypocotyls were 6 mm long, then transferred to the light for 7 d, and averaged. (c,d) Numbers of emergent lateral roots were counted on seedlings grown in vitro directly in light conditions for 10 d and averaged as described in the Methods. At least 30 seedlings of each line were analyzed, the experiments were repeated three times and the data pooled. Error bars indicate standard error; A one-way ANOVA combined with Dunnett’s comparison post-test was used to compare single mutant lines with their respective wild type, and double mutant lines with sur2-1gl1; (*) indicates values significantly different from Col-0 values; (#) indicates values significantly different from Ler values; (o) indicate values that were significantly different from sur2-1gl1 values; ns indicates values not significantly different from Ler (c) or sur2-1gl1 (d) values (\(P < 0.05; n > 90\)).
the cell cycle progression. Nevertheless we have previously shown that jasmonate, through the CORONATIN INSENSITIVE 1 (COI1) signaling pathway, negatively regulates AR initiation in Arabidopsis while others have shown that it promotes LR formation. Therefore, considering the fact that the COP9 signalosome modulates JA responses by physically interacting with the SCF\(^{COI1} \) complex on one hand and that it modulates JA biosynthesis on the other hand, we can speculate that the different CSN subunits may differentially control AR and LR formation by differentially modulating the JA signaling pathway or JA biosynthesis.

Alternatively, several lines of evidence suggest that light-auxin cross-talk takes place during AR development in Arabidopsis. First, in the ago1 mutant, the defect in AR formation correlates with an alteration of auxin homeostasis and a hypersensitivity to light. Second, a model for genetic control of AR formation in Arabidopsis hypocotyls that integrates light and auxin signaling has been published. In addition, previous studies suggest that shoot-localized phytochromes regulate lateral root development, and root-localized phytochromes and cryptochromes regulate phototropic responses and growth of primary roots. Also, it has been shown that light regulates developmental processes at least in part through cross-talk with the phytohormone auxin. Light signaling involves nuclear-cytoplasmic partitioning of phytochromes and negative regulators such as CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), an E3 ubiquitin ligase involved in 26S proteasome-mediated protein degradation. In darkness, COP1 accumulates in the nucleus to degrade transcription factors including HY5, HYH, PHOTOMORPHOGENIC 1 (COP1), an E3 ubiquitin ligase involved in 26S proteasome-mediated protein degradation. The conditions in the controlled environment chambers were as follows: 130 \( \mu \text{m} \) shoots and separated on a 4% agarose gel. The PCR products were digested with HpaI (Fermentas Fast Digest) following the manufacturer’s recommendations and separated on a 4% agarose gel. Alternatively, several lines of evidence suggest that light-auxin cross-talk takes place during AR development. For more general interest, it will provide a tool for a further in-depth study of CSN-independent functions of the CSN4 subunit in Arabidopsis.

Methods

Plant material and growth conditions. The 2035 mutant was identified in a previously described screening. In order to avoid selecting wild-type seedlings due to potential contamination of the mutagenized population with wild-type seeds, the glabra1 mutation was introgressed in the sur2-1 mutant background. The glabra1 mutant was identified in the Versailles collection of T-DNA insertion lines and was therefore in the same genetic background as sur2-1. Homozygote seeds from the double mutant superroot2-1gl1 (sur2-1gl1) (ecotype Wassilewskija, Ws-4) were mutagenized with ethyl methanesulphonate, as described in ref. 51.

The csn4-1 (SALK_043720) and csn4-2 (Salk_053839) segregating lines were described in ref. 17; csn5a-1 (SALK_063436), csn5a-2 (SALK_027705) and csn5b-1 (SALK_007134) were described in ref. 5; csn2-5 was described in ref. 7; csn1-10 was described in ref. 6, and csn3-3 was described in ref. 8. The 2035 mutant is in Wassilewskija (Ws-4) background, the csn2-5 mutant is in a Landsberg erecta (Ler) background while the rest are in a Columbia (Col-0) background. In vitro characterization and auxin quantification were conducted as described previously. For the phenotypic evaluation of soil-grown plants, the seeds were first germinated in vitro and subsequently the seedlings transferred into pots. The plants were then placed in growth chambers in long day (16h light/8h darkness) conditions, at 22°C/18°C (light/dark), 130 \( \mu \text{E m}^{-2} \text{s}^{-1} \) irradiance on average and 60% relative humidity.

For root growth assays, 6-day-old seedlings grown in the light as described in ref. 36 were transferred to the same medium supplemented with various concentrations of 2,4-D, and root growth was measured after an additional 5 days. The conditions in the controlled environment chambers were as follows: 130 \( \mu \text{E m}^{-2} \text{s}^{-1} \) irradiance on average, 16/8 light/dark cycle, 22/15°C, 60% relative humidity. Plates were scanned before and after transfer of the seedling on auxin containing medium. Root length was measured using the ImageJ software package (http://rsb.info.nih.gov/ij/index.html) and ref. 52. Percentage inhibition was calculated by dividing the average growth on 2,4-D containing medium by the average growth on control medium and subtracting this ratio from 100% (n > 25).

Complementation test and genotyping the csn4 alleles. For complementation analyses, homozygous 2035 plants were crossed with heterozygous csn4-1 and csn4-2, respectively. F1 progeny were phenotyped and subsequently F2 progeny were tested, using allele specific PCR primers, as follows. The csn4-1 mutation was genotyped using CSN4-FW1 and CSN4-RV1.1 to test for the presence of the wild-type gene, and LB1.3 and CSN4-RV1 to test for the presence of the T-DNA. The csn4-2 mutation was genotyped using CSN4-FW2 and CSN4-RV2 to test for the presence of the wild-type gene and LB1.3 and CSN4-RV2 to test for the presence of the T-DNA. To genotype the csn4-3 point mutation, newly derived cleaved-amplified polymorphic sequence primers were designed using the dCAPS Finder 2.0 software (ref. 53, http://helix.wustl.edu/dcaps/dcaps.html). Two mismatches (underlined) were introduced in the F primer to incorporate a restriction site in the PCR product of one allele. After amplification, the PCR products were digested with Hpal (Fermentas Fast Digest) following the manufacturer’s recommendations and separated on a 4% agarose gel. The csn4-3 allele yielded two fragments of 150 and 19 bp, while the wild type gave one band of 169 bp. All primers are listed in Supplementary Table S1.
Hypocotyl growth under different light conditions. Seeds were surface sterilized, sown in rows in vitro on media without sugar, stratified for 48 h at 4 °C, then transferred to a plant growth chamber under white light conditions for 4 h to activate germination. Subsequently, five Petri dishes of each light condition/replicate were placed on top of each other, with one chromatography paper layer between them, and wrapped with black plastic leaving the top uncovered (see Supplementary Fig. S2). They were transferred to plant growth cabinets at 20 °C with constant blue (cB) or constant red (cR) light, respectively. The light intensity measured on each layer, bottom to top, was as follows: 1: 3; 11: 41 and 210 μmol m⁻² s⁻¹ irradiance of cR light, and 0.1: 0.4; 2: 10 and 52 μmol m⁻² s⁻¹ irradiance of cB light, respectively. For dark growth conditions, five plates/chamber/replicate were wrapped in three layers of aluminum foil and placed vertically. After 7 d, the seedlings were bended on the surface of the medium, the plates were photographed and hypocotyls measured using ImageJ software (http://rsb.info.nih.gov/ij/index.html). All measurements were performed on three independent biological replicates with a minimum of 40 seedlings each.

Real-time PCR experiments and data analysis. Transcript abundance was assessed by quantitative real-time (RT) PCR as previously described. All quantifications were repeated with three independent biological replicates, using the following standard thermal profile: 10 min at 95 °C, followed by 40 cycles at 95 °C for 10 s, 60 °C for 15 s (except for GH3.5 for which the annealing temperature was 65 °C), and 72 °C for 15 s. The sequences of primers used are presented in Supplementary Table S2. Using the RefFinder: http://www.leonxie.com/referencengene.php, EF1A (At5g60390) has been validated as the most stably expressed gene, of the four tested, and was used to normalize the RT-PCR data. The expression levels were calculated as previously described. Gene expression values are relative to the expression in the wild type, for which the value is set to 1. All RT-PCR results presented are means from three independent biological replicates. For each independent biological replicate, the relative transcript amount was calculated as the mean of three technical replicates, using the method for calculation of standard errors in relative quantification recommended by.

Immunoblot analysis. Total proteins were extracted from 10-day-old light grown seedlings in protein extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP40, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), and Protease Inhibitor cocktail tablets (Roche) 41. A aliquote of 40 μg of total protein for each genotype was loaded and separated by 10% SDS-PAGE gel. Samples were then blotted and used for immunodetection. anti-CSN4 (1:1000) (COP9 signalosome subunits polyclonal antibody) and anti-CUL1 (1:1000) (CUL1 (Arabidopsis thaliana) polyclonal antibody) were purchased at Enzo Life Sciences (http://www.enzolifesciences.com). Goat anti-Rabbit IgG (1:20000) (Agrisera http://www.agrisera.com/en/info/about-agrisera-.html) was used as a secondary antibody.

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D.I.P., M.L.P., A.L., A.M.P. and A.R. performed the experiments; D.I.P. and C.B. designed the experiments and wrote the paper.

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