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4 **Instability of the Arabidopsis mutant *csn5a-2* caused by epigenetic modification of**
5
6 **intronic T-DNA**
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50 Abbreviations: ABRC, Arabidopsis Biological Resource Center; AD, Gal4 activation
51 domain; AP, Alkaline phosphatase; 3-AT, 3-Amino-1,2,4-triazole; BCA, bicinchoninic
52 acid; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; DAG, days after germination; DB,
53 Gal4 DNA-binding domain; NASC, Nottingham Arabidopsis Stock Centre; NBT, nitro-
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4 blue tetrazolium; NPT II, neomycin phosphotransferase II; OD, optical density; RT-
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6 qPCR, reverse transcriptase quantitative polymerase chain reaction.
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10 11 **Abstract** 12 13 14

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16 T-DNA insertion mutants play a crucial role in elucidating Arabidopsis gene function. In
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18 some cases, two or more T-DNA mutants are combined to study genetic interactions
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20 between homologous genes or genes hypothesized to act in the same pathway. We
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22 studied the significance of protein-protein interactions between CSN5A and ROP11 by
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24 crossing three independent *rop11* T-DNA insertion mutants with *csn5a-2*, a partial loss-
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26 of-function intronic T-DNA insertion mutant. The *csn5a-2* single mutant is severely
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28 stunted, but double *csn5a-2 rop11* mutants were rescued and exhibited increased *CSN5A*
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30 transcript and protein levels. The rescued phenotype was maintained in non-Mendelian
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32 fashion when the *csn5a-2* single mutant was re-isolated from the *csn5a-2 rop11-1* double
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34 mutant, and was sensitive to two inhibitors of DNA methylation. Loss of kanamycin
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36 resistance was also observed in re-isolated *csn5a-2*. These findings indicate that the
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38 rescue of *csn5a-2* resulted from a *trans* T-DNA-mediated epigenetic effect on the *csn5a-*
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40 *2* intronic T-DNA, similar to recent reports involving the intronic T-DNA mutants *ag-*
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42 *TD*, *ben1-1*, and *cob-6*. Thus the work reported here provides further support for the
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44 recommendation that mutants created through novel combinations of T-DNA alleles
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46 should be carefully evaluated for evidence of epigenetic modification of T-DNA before
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48 final conclusions are drawn.
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7 **Keywords:**
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9 ROP GTPase; yeast two-hybrid; intronic T-DNA; epigenetic; CSN5A; double mutant
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1. Introduction

We are investigating the roles of protein-protein interactions associated with xylem differentiation. ROP11 (a member of the Rho-like GTPase Of Plant family of small G proteins in Arabidopsis) interacts with MIDD1 to regulate the patterning of secondary cell walls of xylem tracheary elements [1]. ROP11 has additional roles in the regulation of ABA responses [2, 3], cytoskeleton organization and membrane cycling [1, 4, 5]. ROP11 also interacts with the COP9 signalosome subunit CSN5A in yeast two-hybrid (Y2H) assays [6]. There are two CSN5 subunits in Arabidopsis, CSN5A and CSN5B, with CSN5A thought to play the largest role in CSN5 action [7, 8]. The CSN5 subunits of the COP9 signalosome catalyze removal of the ubiquitin-like protein NEDD8/RUB from cullin (CUL) family proteins of cullin-RING E3 ligase complexes, and are therefore crucial to COP9 signalosome activity in the ubiquitin-proteasome pathway [9-11]. CSN5 proteins also interact with and affect the stability of a variety of other proteins not associated with the COP9 signalosome [11]. Thus both ROP11 and CSN5A are involved in a wide range of functions that depend on their interactions with other proteins, but the physiological significance of the ROP11-CSN5A interaction is not yet known. We sought genetic evidence regarding the ROP11-CSN5A interaction by analyzing *rop11 csn5a* double mutants.

CSN5A mutants exhibit severe dwarfing and loss of apical dominance. However, the partial loss-of-function *csn5a-2* mutant phenotype is less severe than that of the null *csn5a-1* mutant, due to the low level of CSN5A protein produced in *csn5a-2* plants [12].

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4 Gusmaroli et al. [12] prepared *cul3a csn5a-2* and *cul3b csn5a-2* double mutants and
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6 found that loss of either CUL3A or CUL3B resulted in higher levels of CSN5A protein
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8 and phenotypic rescue of the *csn5a-2* mutant. Based on these results, we reasoned that if
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10 an interaction between ROP11 and CSN5A affected the abundance or activity of CSN5A,
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12 then loss of *ROP11* expression in the *csn5a-2* mutant background should alter the *csn5a-*
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14 *2* phenotype. We used *rop11* T-DNA insertion mutants to test this hypothesis, and
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16 discovered that introduction of *rop11* alleles into the *csn5a-2* intronic T-DNA mutant
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18 background destabilized the *csn5a-2* phenotype through an epigenetic mechanism that
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20 was independent of *ROP11* loss-of-function, and instead was consistent with *trans* T-
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22 DNA-mediated methylation of the intronic T-DNA.
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31 Similar findings involving epigenetic modification of intronic T-DNA were recently
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33 reported for three other intronic T-DNAs affecting *AGAMOUS*, required for stamen and
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35 carpel formation, (*ag-TD*), *BRI1-5 ENHANCED 1*, involved in brassinosteroid
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37 inactivation (*ben1-1*), and *COBRA*, required for cellulose synthesis, (*cob-6*) [13-15]. In
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39 these reports, as in the case of our findings for *csn5a-2*, gene expression levels repressed
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41 by intronic T-DNA were partially de-repressed following genetic crosses that introduced
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43 new T-DNA inserts for the creation of double or triple mutants. De-repression was
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45 dependent on the addition of T-DNA that contained sequences identical to those in the
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47 intronic T-DNA. A rescued phenotype persisted following re-isolation of the intronic T-
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49 DNA single mutant from the double or triple mutants, suggesting that structural or
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51 epigenetic changes had altered the intronic T-DNA in the presence of additional copies of
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53 T-DNA. Evidence presented in these prior reports in support of *trans* T-DNA-mediated
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methylation of the intronic T-DNA included, 1) loss of NPT II activity (kanamycin resistance) from the re-isolated intronic T-DNA line [13, 14], independent of alteration of *NPT II* gene structure [14], 2) sensitivity of the rescued phenotype to both loss of DNA methyl transferase gene function and treatment with methylation inhibitors 5-azacytidine and zebularine [15], and 3) resistance to bisulfate conversion of cytosine nucleotides in the nopaline synthase promoter (*pNOS*) and adjacent *NPT II* genomic DNA within the intronic T-DNA of re-isolated lines [14]. These reports and our findings for the *csn5a-2* intronic T-DNA mutant should serve to raise awareness of the susceptibility of intronic T-DNA insertion mutants to epigenetic modifications that de-repress gene expression and potentially confound analyses of double or higher order mutants.

2. Materials and methods

2.1 Yeast two-hybrids assays

Yeast transformation was performed according to Walhout and Vidal [16]. Vectors pDEST-AD and pDEST-DB were used for expression of potential interacting proteins in yeast strains Y8800 and Y8930, mating type *MATa* and *MATα*, respectively [17]. The genotype of these strains is: *leu2-3, 112 trp1-901 his3Δ200 ura3-52 gal4Δ gal80Δ GAL2::ADE2 GAL1::HIS3@LYS2 GAL7::lacZ@MET2 cyh2^R*. Due to auto-activation by DB-CSN5A (see section 3.1), *CSN5A* was cloned into pDEST-AD. ROP11 and ROP8 were cloned into pDEST-DB. After mating, diploid cells were selected on plates lacking Leu (-Leu) and Trp (-Trp) for 48 hours. For Y2H, only cells growing on -Leu -Trp plates were further tested for *GAL1::HIS3* reporter gene activation with 5 mM 3-Amino-1,2,4-

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4 triazole (3-AT) and *GAL7::lacZ* reporter gene with X-gal (5-bromo-4-chloro-indolyl- β -
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7 D-galactopyranoside) as the substrate in filter-based assays [18].
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10 11 12 *2.2 Plant materials and growth conditions*

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14 *Arabidopsis thaliana*, ecotype Columbia-0, was used for all experiments involving wild
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16 type (WT) and T-DNA insertion mutant plants. The *CSN5* mutants *csn5a-1*
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18 (SALK_063436), *csn5a-2* (SALK_027705), and *csn5b-1* (SALK_007134) were
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20 described previously [12, 19] and kindly provided by Claus Schwechheimer. *ROP11* T-
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22 DNA insertion lines *rop11-0* (SALK_039681) and *rop11-1* (SALK_013327) were
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24 obtained from ABRC [20]. The *ROP11* T-DNA insertion line *rop11-3* (GABI_345D05)
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26 was obtained from NASC [20]. Plants were grown under long-day conditions (16 h
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28 light/8 h dark) in growth chambers at 22° C. For the kanamycin sensitivity test,
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142 *Arabidopsis* plants were grown on ½ MS plates (2.12 g/L MS salts with macro- and
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144 micronutrients, 10 g/L sucrose, 4 g/L Phytigel) supplemented with 50 mg/L kanamycin.
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146 Surface-sterilized *Arabidopsis* seeds were gently positioned on ½ MS plates, sealed with
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148 parafilm, and incubated at 4° C for at least 2-4 days prior to placing under long-day
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150 conditions at 22° C for plant growth. For DNA methylation inhibitor assays, *Arabidopsis*
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152 plants were grown on ½ MS plates supplemented with 5-azacytidine or zebularine at
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154 concentrations indicated in section 3.6. Seedlings were harvested at 9 DAG for RNA and
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156 protein extraction.

157 158 159 *2.3 PCR-based genotyping*

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161 All *Arabidopsis* T-DNA insertion mutant genotypes were verified by PCR-based
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4 genotyping. For each T-DNA insertion allele, gene specific forward and reverse primers
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6 (Table S1) spanning T-DNA were used in combination with T-DNA left boarder primer
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8 to detect the presence of T-DNA. Genotyping of *CSN5* mutants was performed based on
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10 methods previously described [12]. Primers used to genotype *rop11* mutants are shown in
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12 Table S1.
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19 *2.4 Transcript analysis by RT-qPCR*

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21 Total mRNAs were prepared from Arabidopsis tissue using RNeasy Mini Kit (QIAGEN,
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23 Venlo, Netherlands). About 2 µg of mRNA was reverse transcribed using the High
24
25 Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA).
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27 PCR reactions were performed on an Applied Biosystems 7500 Real-Time PCR System
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29 using *power* SYBR Green PCR Master Mix (ABI, Foster City, CA, USA). *PP2A* gene
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31 transcript was used as the internal control. Three technical replicates for each sample
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33 were run within a 96-well plate, and data shown is representative of at least three
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35 biological replicates. Relative transcript levels were generated by the applied biosystems
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37 7500 software using $2^{(-\Delta\Delta Ct)}$ analysis [21] with the transcript level for WT being set as 1.
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39 Primers used for *ROP11* and *CSN5A* relative transcript level measurements are listed in
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41 Table S1.
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51 *2.5 Western blots*

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53 Approximately 100 mg of 9-day-old Arabidopsis seedlings were ground in liquid
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55 nitrogen and homogenized in 125 µl KEB buffer (25 mM Tris-HCl 7.8, 10 mM MgCl₂, 5
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57 mM EGTA, 2 mM DTT, 10% glycerol, 75 mM NaCl, 60 mM β-glycerophosphate, 0.2%
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NP-40, 0.1 mM Na₃VO₄, 1 mM benzamidine, and 1X EDTA-free protease inhibitor). DTT, β-glycerophosphate, benzamidine and protease inhibitor (Halt Protease Inhibitor Cocktail 100X, Thermo Scientific, Waltham, MA, USA) were freshly added to KEB buffer prior to use. The homogenates were centrifuged twice for 10 min at 14,000 rpm at 4 °C, and after each centrifugation the supernatants were saved into a new tube. Protein concentrations were determined by the BCA assay (Pierce BCA Protein Assay Kit, Thermo Scientific, Waltham, MA, USA).

Total protein was resolved using pre-cast 4-12% polyacrylamide gels (NuPAGE Novex 4-12% Bis-Tris Gel, Life Technologies, Carlsbad, CA, USA). Rabbit polyclonal anti-RPN6 and anti-CSN5 antibodies (Enzo life science, Farmingdale, NY, USA) were used for detection of the loading control, RPN6, and CSN5A protein, respectively. The primary antibody dilution ratio was 1:4000. Goat anti-rabbit IgG-AP (SouthernBiotech, Birmingham, AL, USA) was used as secondary antibody, at a dilution ratio of 1:2000. NBT and BCIP were used for colorimetric detection of AP activity. Results shown are representative of at least two biologically replicated experiments.

2.6 Vector construction and Arabidopsis transformation

The *ROP11* coding region was amplified from Arabidopsis cDNA using primers *tROP11For1* and *tROP11Rev1* (Table S1) and cloned into pENTR/D-TOPO vector (Life technologies, Carlsbad, CA, USA). From this vector the *ROP11* coding region was subcloned into the pFGC5941 binary vector (<http://www.chromdb.org/rnai/pFGC5941.html>) through restriction endonuclease sites

Xho I and *Xma* I. The dominant negative mutant of *CSN5A* (*DN-CSN5A*) included the D175N mutation reported by Gusmaroli et al. [8] and was prepared using PCR-based mutagenesis using primers *CSN5AD175Nfor* and *CSN5AD175Nrev* (Table S1). The mutation was confirmed by sequencing and *DN-CSN5A* was cloned into the pFGC5941 vector through restriction endonuclease sites *Asc* I and *Xma* I.

Arabidopsis transformation was accomplished using the floral dip method [22] with modification. Liquid 2XTY medium (16 g Bacto-tryptone, 10 g Bacto-yeast extract, 5 g NaCl per liter) instead of LB was used to grow *Agrobacterium tumefaciens* strain GV3101 (pMP90) harboring the desired construct. Inflorescences were submerged in the infiltration buffer longer than described (i.e. for 10 min) followed by enclosure in plastic wrap overnight to maintain appropriate moisture for infection. Transformed Arabidopsis plants were then returned to the growth chamber to resume growth until seeds were ready to be harvested.

2.7 Gene identification numbers

CSN5A, AT1G22920; *CSN5B*, AT1G71230; *ROP8*, AT2G44690; *ROP11*, AT5G62880; *PP2A*, At1g13320.

3. Results

3.1 *CSN5A* interacts with *ROP11* in yeast two-hybrid

DB-*CSN5A* auto-activated yeast reporters in Y2H, as reported previously [6] and confirmed by our assays (Fig. 1A), thus limiting the use of *CSN5A* to AD-*CSN5A* only.

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4 As DB-ROP11 did not auto-activate yeast reporters, we used DB-ROP11 and AD-
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6 CSN5A and confirmed the previously reported [6] ROP11-CSN5A interaction (Fig. 1B).
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8 In contrast to ROP11 (a group-II ROP [23]) ROP8 (a group-I ROP) did not interact with
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10 CSN5A in Y2H. This negative Y2H result for DB-ROP8 plus AD-CSN5A shows that
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12 AD-CSN5A is not an auto-activator, further supporting the conclusion that the ROP11-
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14 CSN5A interaction is necessary for activation of yeast reporters.
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21 *3.2 Characterization of ROP11 T-DNA insertion mutants*

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24 We focused on further characterization of the ROP11-CSN5A interaction, due to the
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26 established roles for ROP11 in xylem cell differentiation [1] and the availability of
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28 multiple independent T-DNA insertion mutants for each gene. We reasoned that if the
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30 interaction between ROP11 and CSN5A affects CSN5A function, then loss of ROP11
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32 should alter the phenotype of the weak *csn5a-2* allele, shifting it toward either WT or the
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34 more severe null *csn5a-1* phenotype, depending on the negative or positive influence,
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36 respectively, of ROP11 on CSN5A. To test this hypothesis and study the genetic
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38 interactions between *CSN5A* and *ROP11*, we used three independent T-DNA insertion
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40 mutants affecting *ROP11*, designated *rop11-0*, *rop11-1* and *rop11-3*, to prepare double
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42 *csn5a rop11* mutants.
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51 For *rop11-0*, the T-DNA was mapped to the 5' untranslated region, 46 base pairs
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53 upstream of the *ROP11* initiating Met codon (Fig. 2A). Reverse transcriptase quantitative
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55 PCR (RT-qPCR) performed on cDNA from homozygous *rop11-0* and WT plants showed
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57 that transcript levels were reduced in *rop11-0* by 56% and 46% using primers located at
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the N-terminus or C-terminus, respectively (Fig. 2B). For *rop11-1*, the T-DNA is centrally located in the first intron. No intact *ROP11* cDNA could be detected in the *rop11-1* homozygous line using primer set *yROP11F1* and *tROP11R1* (Fig. 2C). RT-qPCR employing a primer set spanning the T-DNA insertion region (*yROP11F1* with *ROP11R2*) also failed to detect *ROP11* transcripts in the *rop11-1* homozygous line (Fig. 2B). However, primers located at the C-terminus of *ROP11* (*yROP11F2* and *tROP11R1*) detected transcript produced by *rop11-1* at about 42% of the WT level. The presence of C-terminal partial *ROP11* transcripts in *rop11-1* could be caused by a promoter-like sequence in the T-DNA. A chimeric N-terminal *ROP11* transcript was also detected in *rop11-1* using primers *yROP11F1* and *SALKR2* (Fig. 2C). However, due to premature stop codons or frame shifts, neither of the chimeric transcripts amplified from *rop11-1* are likely to yield partial ROP11 proteins. Thus we concluded that the failure to detect full-length *ROP11* transcripts from the *rop11-1* homozygous line (Fig. 2C) indicated it was a null mutant. The T-DNA insertion site for *rop11-3* line is located precisely in the junction of the third intron and fourth exon of *ROP11*. Although full-length *ROP11* transcripts could not be detected from homozygous *rop11-3* cDNA, two distinct PCR products were discovered using *yROP11F1* and the GABI left-border primer *GABIR5* (Fig. 2C). Sequencing of both products showed that the smaller product was an N-terminal cDNA from *ROP11* comprised of the first three exons, while the larger product included the third intron (108 bp) in addition to the first three exons. The presence of these chimeric transcripts suggested that the *rop11-3* line may produce the N-terminal portion of the ROP11 protein, which contains the conserved G1, G2 and G3

domains of ROP GTPases and may fulfill partial function of ROP11. Thus we view *rop11-3* as a potential reduction-of-function line.

3.3 *ROP11* T-DNA insertion mutants rescue the *csn5a-2* phenotype

Two T-DNA insertion mutants for *CSN5A* have been characterized previously: *csn5a-1*, a null mutant, and *csn5a-2*, a reduction-of-function or weak mutant. In the latter line, the T-DNA insertion resides in the fourth intron near the C-terminus, and results in substantial reduction, but not elimination, of *CSN5A* transcript and protein. Hence, the severe stunting caused by loss of *CSN5A* expression in *csn5a-1* is attenuated in the *csn5a-2* mutant, as shown in Figure 3A and reported previously [12, 19], illustrating the high degree of sensitivity of plants to changes in *CSN5A* levels. To investigate the effect of *ROP11*, a *CSN5A*-interacting protein (Fig 1B), on the *csn5a-2* mutant phenotype, we introduced the aforementioned *ROP11* alleles into the *csn5a-2* background by crossing homozygous *csn5a-2* plants with three homozygous *ROP11* T-DNA insertion lines and isolating homozygous double mutants from the F2 populations produced by self-fertilized F1 plants. Consistent with previous investigations of *ROP11* loss-of-function lines [3], none of homozygous *rop11* lines was noticeably different from WT plants (Fig. 3B).

The double mutant *rop11-1 csn5a-2* was produced and studied first because it is null for *ROP11* (Fig. 2). The overall WT-like morphology of the *rop11-1* single mutant contrasts sharply with the severely stunted growth of *csn5a-2* plants (Fig. 3A-C). Surprisingly, *rop11-1 csn5a-2* exhibited a strong rescue phenotype, which is almost indistinguishable from WT plants in the mature stage but is characterized by slightly smaller plants most

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4 evident at earlier developmental stages (compare Fig. 3B with 3C). Additionally, *rop11-1*
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7 *csn5a-2* leaf blades exhibited a more pronounced downward curvature along the midrib
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9 leading to narrower overall leaf morphology when viewed from above. (Fig. 3A).

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14 Next we examined the impact of loss of *CSN5B* function on the *rop11-1 csn5a-2* double
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16 mutant. *CSN5B* encodes an isoform of CSN5A, with which it shares 88% amino acid
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18 identity. CSN5A and CSN5B incorporate into distinct COP9 protein complexes in planta,
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20 but play unequal roles, with CSN5A being the dominant subunit in terms of both protein
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22 abundance and functionality. The null mutant *csn5b-1* has no obvious morphological
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24 phenotype, while the *csn5a-1 csn5b-1* double mutant that is null for both CSN5A and
25
26 CSN5B is lethal in Arabidopsis [12]. On the other hand, the *csn5a-2 csn5b-1* double
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28 mutant is similar to *csn5a-2* and can survive and reproduce, despite the fact that overall
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30 growth is severely compromised [12]. We prepared the *rop11-1 csn5a-2 csn5b-1* triple
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32 mutant and found that it no longer displayed the severe pleiotropic phenotype of *csn5a-2*
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34 *csn5b-1*, although the downward curvature of the leaf blade along the midrib appeared
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36 more pronounced in the *rop11-1 csn5a-2 csn5b-1* triple mutant compared to the *rop11-1*
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38 *csn5a-2* double mutant (Fig. 3A). This experiment revealed that the rescue of the *csn5a-2*
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40 phenotype by the *rop11-1* allele was largely independent of *CSN5B*, but also showed that
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42 *CSN5B* does make a small contribution to normal leaf morphology.

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48 To determine whether the rescuing effect of *rop11-1* on *csn5a-2* required some
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50 expression of CSN5A or could also be observed for null CSN5A mutants, the *rop11-1*
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52 *csn5a-1* double mutant was generated. In contrast to the result with the weak *csn5a-2*
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4 allele, the phenotype of *rop11-1 csn5a-1* was indistinguishable from the *csn5a-1* single
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6 mutant, indicating that the apparent rescue of the *csn5a-2* phenotype by *rop11-1* required
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8 *CSN5A* expression.
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14 The *rop11-0* and *rop11-3* T-DNA insertion lines were also crossed with *csn5a-2*. As
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16 shown in Figure 3B, both *rop11-0 csn5a-2* and *rop11-3 csn5a-2* double mutants
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18 displayed a rescued phenotype, but the complementing powers of these two *ROP11*
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20 mutants were not equivalent to that of the *rop11-1* line. A comparison of 15-day-old
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22 plants from all three double mutants grown under long-day conditions revealed that the
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24 double mutant *rop11-1 csn5a-2* was the most similar to WT, except for the slightly
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26 smaller plant size and aforementioned leaf curvature, while *rop11-3 csn5a-2* double
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28 mutants were most similar to the *csn5a-2* single mutant, including a small proportion of
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30 plants in the population that exhibited only very subtle complementation of the *csn5a-2*
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32 phenotype. The *rop11-0 csn5a-2* double mutant exhibited a degree of rescue that was
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34 intermediate between *rop11-1 csn5a-2* and *rop11-3 csn5a-2*.
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43 3.4 *CSN5A* protein is required for the rescue of the *csn5a-2* phenotype

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45 As illustrated by Figure 3, compared to *rop11-0* and *rop11-3*, the *rop11-1* allele had the
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47 strongest impact on the *csn5a-2* phenotype, but had no effect on the *csn5a-1* phenotype,
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49 indicating that *CSN5A* expression is necessary for the rescue and may be increased in the
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51 presence of *ROP11* T-DNA insertion lines. We investigated the effect of *rop11-1* on
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53 *CSN5A* protein levels by western blotting (Fig. 4). As expected, *CSN5A* protein was
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55 barely detectable in extracts from *csn5a-2*. However, *CSN5A* protein increased in the
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4 *rop11-1 csn5a-2* double and *rop11-1 csn5a-2 csn5b-1* triple mutants compared to *csn5a-*
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7 2. Notably, CSN5A protein level in the *rop11-1 csn5a-2* double mutant was still less
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9 than WT, indicating that the rescue by *ROP11* T-DNA insertion lines is not a full rescue
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11 with regard to CSN5A protein, a conclusion that is consistent with the observation that
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13 *rop11-1 csn5a-2* double mutants exhibited slight deviations from WT plants, i.e., smaller
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15 size of young plants and downward leaf curvature (Fig. 3A). Although the anti-CSN5
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17 polyclonal antibody used for these experiments can detect both CSN5A and CSN5B,
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19 CSN5A accounts for virtually all of the protein detected on blots produced during this
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21 study, as there is little difference between protein abundance in the *rop11-1 csn5a-2*
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23 *csn5b-1* extract (lacking CSN5B) and that in the *rop11-1 csn5a-2* extract, and no CSN5
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25 protein was detected using anti-CSN5 antibody against extracts from the null *csn5a-1*
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27 mutant (Fig. 4). Consequently, protein detected by anti-CSN5 antibody in western blots
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29 presented here is labeled CSN5A.
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38 We confirmed that the rescued phenotype exhibited by *rop11-1 csn5a-2* was dependent on
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40 CSN5A protein function by overexpressing a dominant negative mutant (D175N) of
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42 CSN5A (DN-CSN5A) [8] in the *rop11-1 csn5a-2* background, which resulted in
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44 restoration of the stunted growth typical of the *csn5a-2* mutant (Figs. 5 and S1).
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50 *3.5 Increase in CSN5A transcript levels is independent of ROP11 expression*

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52 To determine whether introduction of *ROP11* T-DNA insertions affected *CSN5A*
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54 transcript abundance as well as protein levels (Fig. 4), we used RT-qPCR to measure
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56 expression of *CSN5A* in double mutants exhibiting the strongest rescue phenotype,
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4 *rop11-0 csn5a-2* and *rop11-1 csn5a-2*. *CSN5A*-specific primers spanning *csn5a-2* T-
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6 DNA insertion site were used to monitor full-length *CSN5A* transcript while eliminating
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8 contamination by partial transcripts either upstream or downstream of the T-DNA. As
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10 shown in Figure 6, relative *CSN5A* transcript levels increased significantly by 3.5-fold
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12 and 2.6-fold for *rop11-1 csn5a-2* and *rop11-0 csn5a-2*, respectively, compared to levels
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14 in *csn5a-2* plants. Although transcript levels for *CSN5A* in double mutants were still well
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16 below those detected in WT (Fig. 6), the observed increase is consistent with the
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18 magnitude of the increase in *CSN5A* protein observed for the *rop11-1 csn5a-2* (Fig. 4)
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20 and shows that introduction of *rop11-0* and *rop11-1* alleles to the *csn5a-2* background is
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22 linked with an increase in production of full-length *CSN5A* mRNA from the *csn5a-2*
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24 allele.
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33 If the loss of *ROP11* expression is specifically required for the increase in *CSN5A*
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35 transcript and protein levels, then increasing *ROP11* expression should reverse the rescue
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37 phenotype and restore the stunted *csn5a-2* phenotype. However, in contrast to the results
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39 for overexpression of *DN-CSN5A*, overexpression of *ROP11* did not restore the *csn5a-2*
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41 phenotype, indicating that the loss of *ROP11* expression in the *rop11-1* mutant was not
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43 necessary for the rescue of the *csn5a-2* phenotype (Fig. 5). In addition to testing the
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45 impact of *ROP11* and *DN-CSN5A* overexpression on the rescued phenotype, we also re-
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47 isolated the *csn5a-2* allele from the *rop11-1 csn5a-2* double mutant background to
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49 determine whether restoration of normal levels of *ROP11* expression would restore the
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51 *csn5a-2* phenotype. We found that the re-isolated *csn5a-2* line retained the rescued
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53 phenotype, i.e., the characteristic *csn5a-2* phenotype did not exhibit Mendelian
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4 segregation after exposure to *rop11-1* (Fig. 5). Together these findings suggest that the
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6 *csn5a-2* allele was epigenetically modified in *trans* by the *ROP11* T-DNA insertions, as
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8 reported recently for other intronic T-DNA insertions [13-15].
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14 As a further test of whether the *csn5a-2* T-DNA had been modified by association with
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16 the *rop11-1* T-DNA insertion, we compared the growth of *csn5a-2* (obtained from the
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18 ABRC), re-isolated *csn5a-2*, and WT Arabidopsis on ½ MS plates containing kanamycin.
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20 We found that while seedlings from the original *csn5a-2* mutant line were resistant to
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22 kanamycin, those obtained by re-isolating *csn5a-2* from the *rop11-1 csn5a-2* double
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24 mutant were sensitive to kanamycin (Fig. 7). The observed loss of kanamycin resistance
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26 for the re-isolated *csn5a-2* mutant suggests that epigenetic modification of this allele is
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28 responsible for the observed increase in *CSN5A* expression and rescue of the stunted
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30 *csn5a-2* phenotype. The loss of kanamycin resistance was not due to structural
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32 modification of the T-DNA, as PCR using primers flanking the *csn5a-2* T-DNA insertion
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34 yielded bands of the same size from *csn5a-2* and re-isolated *csn5a-2* (Fig. S2).
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43 *3.6 The rescued phenotype and increased CSN5A gene expression are sensitive to DNA* 44 45 *methylation inhibitors* 46 47

48 DNA methylation was recently linked to *trans* T-DNA-mediated modifications of
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50 intronic T-DNA [14, 15]. To test whether increased DNA methylation could be a factor
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52 in the rescue of the *csn5a-2* phenotype by *rop11* T-DNA insertion mutants, Arabidopsis
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54 plants were grown on ½ MS medium supplemented with different concentrations of two
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56 methylation inhibitors, 5-azacytidine or zebularine. Selected genotypes grown on ½ MS
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media alone or ½ MS media supplemented with 30 µM 5-azacytidine, 10 µM zebularine, or 30 µM zebularine are shown as representative of effects observed in 3 independent experiments (Figs. 8 and S3).

Neither 5-azacytidine nor zebularine caused abnormal growth of WT plants, when used at the levels shown in Figure 8. In contrast, rescued phenotypes exhibited by *rop11-0 csn5a-2*, *rop11-1 csn5a-2*, double mutants and the *rop11-1 csn5a-2 csn5b-1* triple mutant reverted to the *csn5a-2* phenotype in the presence of inhibitors, consistent with the hypothesis that increased DNA methylation was positively correlated with rescue of the *csn5a-2* phenotype. That *rop11-1 csn5a-2 csn5b-1* triple mutants were more sensitive to zebularine treatment than double mutants (Fig. 8D) again reveals the contribution of *CSN5B* to the rescue phenotype of *rop11-1 csn5a-2* plants, as noted above (Fig. 3A). Plants were affected by 30 µM 5-azacytidine to a lesser extent, possibly due to instability of 5-azacytidine in aqueous solutions [24]. In the same experiments, methylation inhibitors did not affect corresponding *ROP11* single mutants, *rop11-0* or *rop11-1* (Fig. S3).

To determine whether methylation inhibitor treatments also led to reduction in *CSN5A* transcript and protein level, we performed RT-qPCR and western blot analyses of plants treated with DNA methylation inhibitor. We chose 30 µM zebularine for these studies, as this concentration proved most effective at promoting reversion to the *csn5a-2* phenotype (Fig. 8). Exposure to the methylation inhibitor was associated with a reduction in *CSN5A* transcript in *rop11-0 csn5a-2* and *rop11-1 csn5a-2* (Fig. 9A). *CSN5A* expression in WT

was not significantly affected by drug treatment. Similarly, zebularine treatment was correlated with reduced CSN5A protein levels in *rop11-1 csn5a-2*, and *rop11-0 csn5a-2* double mutants, but did not affect CSN5A protein in WT, *rop11-1*, or *rop11-0* plants (Fig. 9B). CSN5A protein was slightly reduced by zebularin treatment in the *csn5a-2* single mutant, consistent with the slight non-significant reduction in *CSN5A* expression under the same conditions (Fig. 9A). Thus the observed reductions in *CSN5A* transcript and protein levels associated with zebularine treatment were correlated with reversion of treated plants to the *csn5a-2* phenotype (Fig. 8). These results obtained through the use of methylation inhibitors are similar to those reported by Xue et al. [15] for the *epicob-6* phenotype and suggest that the rescue of the *csn5a-2* stunted growth phenotype by *rop11-0* and *rop11-1* T-DNA insertion mutants is at least partly due to methylation affecting the *csn5a-2* intronic T-DNA.

4. Discussion

The Arabidopsis ROP GTPase ROP11 is one of more than 100 proteins recently reported to interact with the COP9 signalosome subunit CSN5A [6]. The *csn5a-2* mutant produces a greatly diminished amount of CSN5A protein compared to WT and exhibits a severe phenotype that is responsive to small changes in CSN5A protein level [12].

Consequently, the *csn5a-2* mutant is potentially a sensitive reporter for determining whether a CSN5A-interacting protein or pathway influences CSN5A protein abundance

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4 or activity. Based on this rationale we combined *ROP11* loss-of-function mutants with
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7 *csn5a-2* to evaluate the impact of ROP11 on CSN5A.
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11 Initial characterizations of three double mutants comprised of independent *ROP11* T-
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13 DNA insertion mutants and *csn5a-2* suggested that ROP11 was a negative regulator of
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15 CSN5A, i.e., loss of *ROP11* function partially rescued the severe *csn5a-2* phenotype.
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17 Similarly, Gusmaroli et al. [12] reported that both the *cul3b csn5a-2* and *cul3a csn5a-2*
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19 double mutant lines rescued the phenotype of *csn5a-2*, and the rescue was correlated with
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21 an increase in CSN5A protein levels, as we found for the rescued *rop11-0 csn5a-2* and
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23 *rop11-1 csn5a-2* mutants described here (Figs. 4 and 9). Furthermore, we showed that
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25 the rescued phenotype was dependent on CSN5A protein function, as overexpression of a
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27 dominant negative CSN5A protein (DN-CSN5A) in the *rop11-1 csn5a-2* double mutant
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29 background reversed the rescued phenotype (Fig. 5). However, additional
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31 characterization of *rop11 csn5a-2* double mutants revealed two key differences between
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33 the results from the CUL3-CSN5A and ROP11-CSN5A investigations. First, *CSN5A*
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35 transcript levels increased in the *rop11 csn5a-2* double mutants compared to the *csn5a-2*
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37 single mutant (Fig. 6), whereas Gusmaroli et al. [12] found no differences in *CSN5A*
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39 transcript levels between the *csn5a-2* and *cul3 csn5a-2* mutants, albeit by using less-
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41 sensitive semi-quantitative RT-PCR compared to our RT-qPCR measurements. Second,
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43 *csn5a-2* re-isolated from the *rop11-1 csn5a-2* background continued to exhibit the
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45 rescued phenotype, i.e., the stunted *csn5a-2* phenotype did not exhibit Mendelian
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47 segregation after exposure to the *ROP11* T-DNA insertions (Fig. 5). Gusmaroli et al. [12]
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49 did not report loss of Mendelian segregation of the *csn5a-2* phenotype in their CUL3-
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CSN5A research, despite using a *csn5a-2* single mutant that was re-isolated from segregating F2 populations created for production of the SALK T-DNA double mutants. In a related finding, overexpression of *ROP11* in the *rop11-1 csn5a-2* double mutant also failed to reverse the rescue and restore the *csn5a-2* phenotype (Fig. 6), further supporting the conclusion that loss of *ROP11* function was not necessary for the rescued phenotype.

The non-Mendelian segregation of the *csn5a-2* phenotype and its overall independence from *ROP11* expression levels suggest that the *csn5a-2* intronic T-DNA was suppressed in *trans* by introduction of the second site SALK T-DNAs, *rop11-0* and *rop11-1*, and to a lesser extent by the GABI T-DNA, *rop11-3*, which shares only the *35S* promoter with SALK T-DNAs and not the *NPT II* gene that confers kanamycin resistance. That the re-isolated *csn5a-2* line was no longer resistant to kanamycin is further evidence of *trans* modification of the intronic *csn5a-2* T-DNA affecting sequences associated with the *NPT II* sequence (Fig. 7).

Three other examples of epigenetic gain-of-function phenotypes involving intronic T-DNA shared characteristics with those reported here for *csn5a-2* [13-15]. In each of these cases, gene expression levels repressed by intronic T-DNA were partially de-repressed following genetic crosses that introduced new T-DNA inserts for the creation of double or triple mutants. De-repression was associated with the addition of T-DNA inserts that contained sequences identical to those in the intronic T-DNA. A rescued phenotype persisted following re-isolation of the intronic T-DNA single mutant from the double or triple mutants, implicating structural or epigenetic changes to the intronic T-

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4 DNA in the presence of additional copies of T-DNA. Similar to findings by Xue et al.
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6 [15] regarding restoration of the *cob-6* phenotype, we found that treatment of double
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8 mutants with either 5-azacytidine or zebularine restored the characteristic *csn5a-2*
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10 phenotype (Fig. 8). In correlation with this biochemical phenocopy of *csn5a-2*, treatment
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12 with 30 μ M zebularine reduced both CSN5A transcript and protein levels in double
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14 mutants (Fig. 9). Together these observations are consistent with the necessity of DNA
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16 methylation for the rescued phenotype exhibited by *rop11 csn5a-2* double mutants.
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24 The mechanism behind the *trans* T-DNA-mediated de-repression of *CNS5A* expression in
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26 *csn5a-2* is not yet known. Based on a mechanism proposed Gao and Zhao [13] for *trans*-
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28 interaction between T-DNA insertions, it is possible that intronic *csn5a-2* T-DNA
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30 reduces *CSN5A* expression due to the formation of dsRNA involving the *NPT II* coding
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32 sequence and a long *csn5a-2* transcript which includes *NPT II* T-DNA sequence
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34 transcribed from the opposite direction. This dsRNA may promote transcript degradation
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36 or more directly interfere with processing of the *CNS5A* transcript. If suppression of
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38 *CSN5A* in *csn5a-2* is dependent on such dsRNA, then transcripts from a second-site *NPT*
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40 *II*-containing T-DNA (i.e., *rop11-0* or *rop11-1* T-DNA insertion mutants) could inhibit
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42 formation of this dsRNA and thereby increase *CNS5A* expression from the *csn5a-2* allele.
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47 The aforementioned dsRNA is also consistent with a role for DNA methylation [25].
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51 Guide siRNAs derived from dsRNA comprised of *NPT II* coding sequence may direct
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53 methylation of *NPT II* coding sequence, while those from *pNOS* (the promoter of *NPT II*
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55 expression in the T-DNA construct) may direct *NPT II* promoter methylation, as shown
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57 in the case of *trans* T-DNA-mediated de-repression of the *ben1-1* allele [14]. Both of
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4 these possible methylation targets are consistent with the observed loss of kanamycin
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6 resistance following the creation of *rop11 csn5a-2* double mutants and inheritance of the
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8 rescue phenotype in *csn5a-2* re-isolated from double mutants. Moreover, silencing of
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10 *NPT II* expression through DNA methylation would also contribute to de-repression of
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12 *CSN5A* expression in *csn5a-2* by reducing the abundance of *NPT II* transcripts that could
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14 form duplexes with long *csn5a-2* transcripts containing the *NPT II* sequence.
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21 Similar *trans*-interactions and resulting DNA methylation are possible for exonic T-DNA
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23 insertions. However, unlike the effects on intronic T-DNA noted here for *csn5a-2* and
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25 previously for *ag-TD*, *ben1-1*, and *cob-6* intronic T-DNA insertion mutants [13-15], the
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27 outcome of such epigenetic changes in exons is not expected to lead to suppression of the
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29 original mutant phenotype, as the coding sequence would remain disrupted by the exonic
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31 T-DNA insertion. Interestingly, DNA methylation in gene bodies affects alternative
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33 splicing [26], suggesting that *trans* T-DNA-mediated methylation of T-DNAs may lead
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35 to production of novel splice variants, which may result in splicing out of exons that
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37 harbor T-DNAs. Further investigation is needed to determine the effects of *trans* T-DNA-
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39 mediated modifications of exonic T-DNA insertions.
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48 In summary, the non-Mendelian segregation of the *csn5a-2* phenotype following
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50 exposure to a second-site T-DNA insert and the loss of kanamycin resistance in the re-
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52 isolated *csn5a-2* mutant coupled with the sensitivity of the rescued phenotype to DNA
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54 methylation inhibitors indicates that a *trans* (*rop11*) T-DNA-mediated increase in *CSN5A*
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gene expression and not direct interactions between CSN5A and ROP11 proteins was responsible for the rescue of the *csn5a-2* phenotype.

To estimate the prevalence of intronic T-DNAs in research involving the production of T-DNA-based double mutants, we conducted a search in *PubMed* (<http://www.ncbi.nlm.nih.gov/pubmed/>) using the terms “double mutant T-DNA”. This search returned 229 citations. Among these were 73 investigations (published from 2002 through 2014) using double or higher-order mutants created by crossing two or more T-DNA lines involving at least one intronic T-DNA. Intronic T-DNAs, therefore, are important components of a substantial number of double mutant studies. However, other than the aforementioned recent examples of epigenetic modification of intronic T-DNA [13-15], no publications retrieved by our query in *Pubmed* made direct reference to rescued phenotypes appearing in double mutants or de-repression of genes containing intronic T-DNAs. Indeed, it is clear from prior work with the *csn5b-1 csn5a-2*, *cul3a csn5a-2*, and *cul3b csn5a-2* double mutants [12], which did not exhibit non-Mendelian rescue phenotypes, that not all T-DNA inserts sharing identical backbone sequences (e.g. SALK T-DNA lines) will promote *trans* T-DNA-mediated modification. Thus additional work is needed to identify all features that are necessary and sufficient to lead to *trans* T-DNA-mediated silencing of intronic T-DNAs. Until such knowledge is available and in light of our findings for *csn5a-2* and those recently reported for other double mutants involving intronic T-DNAs [13-15], we conclude that such mutants should be examined for evidence of epigenetic modification of intronic T-DNA before final conclusions are drawn.

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Figure Legends

Figure 1. CSN5A interacts with ROP11.

(A) Yeast cells transformed for expression of *DB-CSN5A* or *DB-ROP11* to test for auto-activation or DB vector only. (B) Yeast cells transformed for expression of *AD-CSN5A* plus *DB-ROP11* or *DB-ROP8* as indicated. The *AD-CSN5A* plus *DB-ROP8* combination serves as a control for auto-activation by AD-CSN5A protein. -L-T, media lacking Leu and Trp; -L-T-H + 5 mM 3-AT, media lacking Leu, Trp, and His and supplemented with 5 mM 3-AT. LacZ reporter, yeast cells selected on -L-T and supplied with X-gal.

Figure 2. Characterization of *ROP11* T-DNA insertions

(A) Schematic of *ROP11* structure and positions of three T-DNA insertions. Untranslated and translated exon regions are represented by gray and black boxes, respectively. White boxes represent introns. Introns and exons are drawn to scale. Triangles show the position of T-DNA insertions. Arrows indicate the locations and orientations of primers used. (B) *ROP11* transcript levels in *rop11-0* and *rop11-1* homozygous plants relative to WT. (C) RT-PCR analysis of *rop11-1* and *rop11-3* homozygous mutants. Actin primers were used as a control for RT-PCR (C). Asterisk in top row (yROP11F1+GABIR5) marks chimeric intron-containing *ROP11* transcript produced by *rop11-3*.

Figure 3. The phenotype of *csn5a-2* was rescued by *ROP11* T-DNA insertion mutants

(A) Arabidopsis WT, single, double, and triple mutants grown under long-day conditions for 23 days. (B) Arabidopsis WT, single, and double mutants grown under long-day

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4 conditions for 15 days. (C) Comparison of single and double mutants grown under long-
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6 day conditions for 45 days. Genotype is indicated in lower left of each image. Bar
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8 within each WT image in panels (A-C) is 5 cm and is for all images within the respective
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10 panel. Inset images (A) are magnified 2.5-fold.
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16 **Figure 4.** Western blot analysis of CSN5A protein levels
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18 Polyclonal anti-CSN5 antibody was used to detect CSN5 protein. CSN5 protein (the sum
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20 of CSN5A and CSN5B) is barely detectable in *csn5a-2* plants, while none is detected in
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22 *csn5a-1* plants, indicating that the anti-CSN5 antibody detected only CSN5A in this
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24 study. CSN5A protein levels in the *csn5a-2* mutant are increased in the presence of the
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26 *rop11-1* allele (*rop11-1 csn5a-2* and *rop11-1 csn5a-2 csn5b-1*). Equal protein loading
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28 was confirmed using anti-RPN6 antibody.
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36 **Figure 5.** Rescue of the *csn5a-2* phenotype depends on CSN5A protein but not on loss of
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38 *ROP11* gene expression.
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40 Overexpression of a dominant negative mutant of CSN5A in the *rop11-1 csn5a-2*
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42 background (*rop11-1 csn5a-2 DN-CSN5A*) restored the stunted *csn5a-2* phenotype. In
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44 contrast, restoring *ROP11* expression levels by re-isolating *csn5a-2* from *rop11-1 csn5a-*
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46 *2 (csn5a-2 Re-isolated)* or overexpression of *ROP11 (rop11-1 csn5a-2 ROP11-OX)* failed
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48 to reverse the rescue and restore the characteristic *csn5a-2* phenotype. Plant genotype is
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50 indicated in the lower left of each panel. Bar within wild type (WT) panel is 5 cm and is
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52 for all panels. All plants shown were grown for 45 days under long-day conditions.
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Figure 6. Analysis of relative *CSN5A* transcript levels in *csn5a-2* single mutant and double mutant rescued lines

Relative *CSN5A* transcript levels ($\pm$ SD, n=3) in WT, *csn5a-2*, *rop11-1 csn5a-2*, and *rop11-0 csn5a-2*. Primers flanking the *csn5a-2* T-DNA insertion region were used to measure *CSN5A* transcript levels relative to WT. Transcript level of WT was set to 1. Asterisks indicate significant differences between double mutant rescued lines and *csn5a-2* (*, $P < 0.01$; Student's t-test).

Figure 7. Loss of kanamycin resistance in re-isolated *csn5a-2*

(A) WT seedlings are sensitive to kanamycin. (B) *csn5a-2* mutant seedlings are resistant to kanamycin, and produce several green leaves. (C) Seedlings of the *csn5a-2* mutant re-isolated from the *rop11-1 csn5a-2* double mutant are no longer kanamycin resistant and produce only one set of small, bleached true leaves. Three-week-old seedlings representative of two independent experiments are shown. Bar in (A) is 2 mm for all panels.

Figure 8. Reversion to the *csn5a-2* phenotype in media supplemented with DNA methylation inhibitors

Selected double and triple mutants, indicated at top of figure, were grown in MS media alone (A) or MS media supplemented with 30 μ M 5-azacytidine (B), 10 μ M zebularine (C), or 30 μ M zebularine (D). Micrographs were taken 7 days after germination.

Figure 9. CSN5A transcript and protein levels in zebularine-treated plants

(A) Relative *CSN5A* transcript levels were determined as for Figure 6. Asterisks indicate significant decreases in expression for plants treated with 30 μ M zebularin compared to untreated plants (*, $P < 0.05$; Student's t-test). (B) CSN5A protein was barely detectable in extracts from *csn5a-2* plants. Relative to the *csn5a-2* single mutant, double mutants exhibited increased levels of CSN5A protein, which was reduced by 30 μ M zebularine treatment (+). Levels of CSN5A protein in *rop11* mutants or WT plants were not affected by 30 μ M zebularine. Anti-RPN6 antibody was used to confirm equal loading.

Figure 1

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