Reversion of the *Arabidopsis rpn12a-1* Exon-Trap Mutation by an Intragenic Suppressor that Weakens the Chimeric 5' Splice Site

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Reversion of the *Arabidopsis rpn12a-1* exon-trap mutation by an intragenic suppressor that weakens the chimeric 5’ splice site [v2; ref status: indexed, http://f1000r.es/18y]

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Abstract

**Background:** In the *Arabidopsis* 26S proteasome mutant *rpn12a-1*, an exon-trap T-DNA is inserted 531 base pairs downstream of the *RPN12a* STOP codon. We have previously shown that this insertion activates a STOP codon-associated latent 5’ splice site that competes with the polyadenylation signal during processing of the pre-mRNA. As a result of this dual input from splicing and polyadenylation in the *rpn12a-1* mutant, two *RPN12a* transcripts are produced and they encode the wild-type *RPN12a* and a chimeric *RPN12a-NPTII* protein. Both proteins form complexes with other proteasome subunits leading to the formation of wild-type and mutant proteasome versions. The net result of this heterogeneity of proteasome particles is a reduction of total cellular proteasome activity. One of the consequences of reduced proteasomal activity is decreased sensitivity to the major plant hormone cytokinin.

**Methods:** We performed ethyl methanesulfonate mutagenesis of *rpn12a-1* and isolated revertants with wild-type cytokinin sensitivity.

**Results:** We describe the isolation and analyses of suppressor of *rpn12a-1* (*sor1*). The *sor1* mutation is intragenic and located at the fifth position of the chimeric intron. This mutation weakens the activated 5’ splice site associated with the STOP codon and tilts the processing of the *RPN12a* mRNA back towards polyadenylation.

**Conclusions:** These results validate our earlier interpretation of the unusual nature of the *rpn12a-1* mutation. Furthermore, the data show that optimal 26S proteasome activity requires *RPN12a* accumulation beyond a critical threshold. Finally, this finding reinforces our previous conclusion that proteasome function is critical for the cytokinin-dependent regulation of plant growth.
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Introduction

The 26S proteasome (26SP) is a multisubunit protease responsible for the degradation of proteins that are covalently labeled with a polyubiquitin (Ub) chain via the combined action of Ub activating enzymes, Ub conjugating enzymes and Ub ligases. The 26SP is localized in the cytosol and the nucleus, and it degrades proteins involved in many signaling and metabolic pathways. The 26SP is also essential for the destruction of misfolded proteins that are generated by mistranslations and during stress.

Studies with proteasome mutants in Arabidopsis have revealed that the 26SP is required for both male and female gametogenesis, confirming its essential role in plant growth and development. Partial loss-of-function mutants, on the other hand, have been indispensable for uncovering pathways in which key components are regulated by proteasome-dependent degradation.

The rpn12a-1 mutant, which carries an insertion in the RPN12a gene (At1g64520) encoding the regulatory particle non-ATPase subunit (RPN) 12a, was isolated from a collection of exon-trap lines. These lines were generated by transforming Arabidopsis plants (C24 accession) with a T-DNA construct that contains a promoterless neomycin phosphotransferase gene (NPTII) without a starting methionine which is preceded by a 3′ splice site of the first intron of the apurinic endonuclease (APR) gene. Kanamycin-resistant exon-trap lines are therefore predicted to have the APR-NPTII construct inserted downstream of an active promoter either in frame with the coding region or in a position that allows the formation of a novel, chimeric intron. The rpn12a-1 mutation is unusual because the T-DNA is inserted downstream of the RPN12a gene, and both the full-length RPN12a cDNA and a chimeric RPN12a-NPTII cDNA are produced. This suggested that two types of cis signals involved in the pre-mRNA processing of RPN12a are competing. Because the wild-type transcript is produced in the mutant and is stable enough to be detected using routine RNA analytical procedures, the poly(A) signal of the RPN12a gene must be intact and active. On the other hand, since a chimeric RPN12a-NPTII transcript is also produced, the 3′ splice site of the inserted T-DNA must have recruited a latent 5′ splice site in the RPN12a gene. We have previously shown that this predicted latent 5′ splice site is STOP codon-associated, and that the pre-mRNA splicing of the chimeric intron leads to the production of the fusion mRNA. As a result of the action of these two opposing pre-mRNA processing mechanisms, one part of the mRNA species transcribed from the mutant RPN12a gene is translated into a functional RPN12a protein, and the other is translated into a chimeric RPN12a-NPTII fusion protein. Because both RPN12a forms are incorporated into the 26SP, the total proteasome activity in these mutant seedlings is reduced, but not abolished.

The reduction of 26SP activity in rpn12a-1 caused a pleiotropic phenotype, which included altered responses to cytokinins. Cytokinins are plant hormones that are essential for every aspect of growth and development. For example, cytokinins control the development of meristems and vasculature, and play an important role in senescence and nutrient allocation. To gain better insight into the cytokinin insensitivity of rpn12a-1 seedlings, we screened for suppressor mutants that have a wild-type cytokinin growth response. Here we describe the intragenic suppressor of rpn12a-1 (sor1) that disrupts the latent 5′ STOP-associated splice site. Sor1 reduced the expression of the RPN12a-NPTII fusion mRNA with a concomitant increase in RPN12a transcript level. As a result, RPN12a accumulation in sor1 seedlings was identical to the wild-type and was accompanied by wild-type cytokinin sensitivity. These results validate our transcript processing interpretation of the rpn12a-1 exon-trap effect and accentuate the importance of optimal RPN12a expression for cytokinin signaling.

Materials and methods

Plant material and growth conditions

The Arabidopsis thaliana rpn12a-1 mutant in the C24 background was described by us previously. To grow plants on soil and in axenic cultures, seeds were surface-sterilized in 70% ethanol followed by 50% bleach and plated on MS/2 medium that contained half-strength MS salts (pH 5.7, Sigma, St. Louis, MO) and 1% (w/v) sucrose. The seeds were kept for 4 days in darkness at 4°C, and either plated on MS/2 or on soil (Miracle-Gro potting mix: Perlite at 1:1 ratio). Plants were grown in continuous light at 22°C.

EMS mutagenesis and screening for rpn12a-1 suppressors

The rpn12a-1 seeds were pre-incubated in 1.0% KCl for 12 hours, and then mutagenized for 5 hours in 100 mM sodium phosphate buffer (pH 5) containing 5% DMSO and 80 mM ethyl methanesulfonate (EMS; Sigma-Aldrich, St. Louis, MO). Seeds were washed twice in 100 mM sodium thiosulphate and then twice in distilled water. Seeds were incubated and chilled in 0.1% agar and sown directly to soil. All the seeds in the M2 generation were pooled upon harvest, surface-sterilized and plated on the MS/2 medium containing 0.1 µM kinetin (6-furfurylaminopurine; obtained from Duchefa Biochemie by Gold Biotechnology, St. Louis, MO, USA). The putative suppressor mutants were transferred from the selection medium onto MS/2 medium to allow recovery, and were then transferred to soil.

Phenotypic analyses of sor1

Cytokinin treatments were as previously described. For fresh weight analyses, seedlings were germinated and grown on kanamycin-containing media, and their weight was measured in pools of 5 seedlings after 24 days of growth. Kanamycin monosulfate was obtained from Gold Biotechnology.
Expression analyses

Total RNA was isolated from Arabidopsis seedlings grown in liquid Gamborg’s B5 medium with 1% sucrose (pH 5.7) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The iScript kit (BioRad, Hercules, CA, USA) and 1 µg of TURBO DNase (Ambion, Austin, TX, USA) pre-treated total RNA was used for the synthesis of the first-strand cDNA. For the RT-PCR experiments, the primers used for the amplification of wild-type cDNA fragment (306 bp in length) were F1: 5'‐GGGTCCTATACGCTCTCGGCCGTAAGGCTAG-3' and R1: 5'‐ATACGCTCCAGGTCCCTGGGTAAGGCTAG-3'. The RPN12A‐NPTII fusion transcript fragment was amplified with F1 and NPTII primer R2: 5’‐CCCCCTGGTGACCACGCCGGAAACA-3’. PBA1 (At4g31300) was amplified using forward and reverse primers that contained the first and last 25 bp of the cDNA. The primer set used to amplify the Arabidopsis elongation factor 1-α (EF-1-α: At5g60390) was previously described. For the quantitative RT-PCR (qPCR), primers were designed using RealTime PCR tool (Integrated DNA Technologies, Coralville, IA, USA). The RPN12a fragment was amplified using qRPN12a F 5’‐AGTGGACAGAGATCAAGGGCCG‐3' and qRPN12a R 5’‐TCGCCTGGTTTGCAGCTTAG-3’ primers. The RPT2a (At4g29040) fragment was amplified by using 5’‐AATCGCAAGATCGGAAAATCT‐3' and 5’‐TCGGCACACAACCTTCTCCATCA-3' as F and R primers, respectively. Previous analyses have shown that the best reference gene for the qPCR analyses of proteasome mutants is ACT2 (At3g18780)23. The qPCR assays were done as previously described23.

For immunoblotting analyses, total proteins were isolated, separated and transferred to nitrocellulose membranes as described. For the RT-PCR experiments, the primers used for the amplification of wild-type cDNA fragment (306 bp in length) were F1: 5’‐GGGTCCTATACGCTCTACACGCTAAGGCTAG-3' and R1: 5’‐ATACGCTCCAGGTCCCTGGGTAAGGCTAG-3’. The RPN12A‐NPTII fusion transcript fragment was amplified with F1 and NPTII primer R2: 5’‐CCCCCTGGTGACCACGCCGGAAACA-3’. PBA1 (At4g31300) was amplified using forward and reverse primers that contained the first and last 25 bp of the cDNA. The primer set used to amplify the Arabidopsis elongation factor 1-α (EF-1-α: At5g60390) was previously described. For the quantitative RT-PCR (qPCR), primers were designed using RealTime PCR tool (Integrated DNA Technologies, Coralville, IA, USA). The RPN12a fragment was amplified using qRPN12a F 5’‐AGTGGACAGAGATCAAGGGCCG‐3' and qRPN12a R 5’‐TCGCCTGGTTTGCAGCTTAG-3’ primers. The RPT2a (At4g29040) fragment was amplified by using 5’‐AATCGCAAGATCGGAAAATCT‐3' and 5’‐TCGGCACACAACCTTCTCCATCA-3' as F and R primers, respectively. Previous analyses have shown that the best reference gene for the qPCR analyses of proteasome mutants is ACT2 (At3g18780)23. The qPCR assays were done as previously described23.

For immunoblotting analyses, total proteins were isolated, separated and transferred to nitrocellulose membranes as described. Rabbit polyclonal anti-RPN12a and anti-PBA1 antibodies (used at 1:1000 dilution) were purchased from Invitrogen (Carlsbad, CA, USA). The rabbit, polyclonal anti-NPTII antibodies (used at 1:1000) were obtained from Abcam (Cambridge, MA, USA).

Analyses of the sor1 mutation

Genomic DNA fragments from rpn12a-1 and sor1 were amplified using F1 and R2 primers and sequenced using dye-termination chemistry (Perkin-Elmer, Foster City, CA, USA) at Advanced Genetic Technologies Center (AGTC, KY, USA). Sequences were analyzed using Vector NTI Suite (Invitrogen, Carlsbad, CA).

Results and discussion

Isolation of an intragenic rpn12a-1 suppressor

To obtain rpn12a-1 suppressors, we mutagenized seeds with EMS and plated ~50,000 M2 seeds on a medium with 0.1 µM of the cytokinin kinetin. Because wild-type plants grown on 0.1 µM kinetin are chlorotic and smaller than the wild-type and rpn12a-1 seedlings, we isolated self-pollination. We isolated several classes of candidate mutants with varying degrees in rpn12a-1 suppression. However, only one of these mutant lines displayed a near-complete reversion to the wild-type phenotype. Here we describe the molecular analyses of this line that we named suppressor of rpn12a-1 (sor1).

Analyses of the M3 generation showed that in suppressor of rpn12a-1 (sor1), all visible phenotypes of rpn12a-1 were reverted back to the wild-type (Figure 1). For example, the rpn12a-1 mutant has a smaller rosette than the wild-type and a reduced leaf initiation rate34. The sor1 plants had a leaf number and rosette size similar to the C24 wild-type plants (Figure 1). The sor1 mutant plants also displayed wild-type sensitivity to cytokinin. After three weeks of growth on a medium with 0.2 µM kinetin, both wild-type and sor1 seedlings were chlorotic and their growth was severely inhibited, while the rpn12a-1 seedlings were green and larger (Figure 1).

Next, we analyzed the kanamycin (Km) resistance of the sor1 mutant line. The Km resistance of the rpn12a-1 mutant is completely linked to the proteasome-related phenotypes and thus, all the progeny of a plant homozygous for the rpn12a-1 mutation should be Km resistant. All sor1 seedlings were indeed resistant to Km, but the levels of resistance were significantly lower compared to rpn12a-1 (Figure 2). While Km did not affect the growth of rpn12a-1 seedlings, both root and shoot growth of sor1 were partially inhibited (Figure 2). We did not observe any attenuation of Km resistance over several generations, a phenomenon that has been documented for a number of T-DNA insertion mutant collections (see also the Salk Institute Genomic Analysis Laboratory Arabidopsis sequence indexed T-DNA insertion Project FAQ). An explanation for the change in Km tolerance in sor1 is that the mutation affects the expression of the NPTII gene which is an integral part of the exon-trap (Figure 3a and Babiyuchk et al. 199735). When the sor1 mutant was outcrossed to the C24 wild type, none of the plants of the F2 population displayed an rpn12a-1 phenotype, indicating that sor1 is intragenic and tightly linked with the rpn12a-1 mutation.

sor1 suppresses the accumulation of the RPN12a-NPTII fusion transcript

To obtain further insight into the nature of the sor1 mutation, we analyzed the expression of the RPN12a gene and the accumulation of the RPN12a protein. RT-PCR analyses showed that in sor1, the RPN12a-NPTII fusion transcript was not detectable and that the RPN12a cDNA level was comparable to the wild type (Figure 3b). Quantitative RT-PCR (qPCR) analyses confirmed that there was no statistically significant difference between RPN12a levels in sor1 and the wild
Immunoblotting analyses using anti-RPN12a antibodies showed that the sor1 mutant does not accumulate the RPN12a-NPTII fusion protein (Figure 4). The RPN12a abundance in sor1 was increased compared to rpn12a-1 and similar to the wild-type. We were also unable to detect the RPN12a-NPTII fusion in sor1 by using

Figure 2. The sor1 mutation leads to a partial loss of kanamycin resistance. (a) Wild-type (C24), rpn12a-1 and sor1 seeds were sown and grown on MS/2 media containing 35 µg/ml kanamycin (Km). Representative plants were photographed after two weeks of growth. (b) Fresh weight (FW) of seedlings grown on Km media was measured after two weeks of growth. FW of the wild-type plants grown on control MS/2 media was calculated as 100%. Seedlings were measured in pools of five, and mean ± SD is presented (n=7).

Reductions in proteasome activity typically lead to the activation of a feedback mechanism that induces the transcription of proteasome subunit genes. This mechanism is operational in all eukaryotes, including yeasts, Drosophila, mammals and plants7,24-28. Due to this global feedback up-regulation of 26SP subunit genes, the 20S proteasome subunit β1 (PBA1) and 26SP regulatory particle subunit RPT2a transcripts were more abundant in rpn12a-1 compared to the wild type (Figure 3b and 3c). RT-PCR analyses suggested and qPCR analyses confirmed that the proteasome subunit transcript levels in sor1 were reduced compared to rpn12a-1, but still increased compared to the wild-type (Figure 3b and 3c), indicating that the sor1 mutation did not lead to a complete suppression of the rpn12a-1 mutation.

Figure 3. The sor1 mutation reduces the expression of the RPN12a-NPTII fusion transcript. (a) Simplified schematic representation of the RPN12a gene and the inserted T-DNA in the rpn12a-1 mutant15. The T-DNA contains the first intron and second exon of the apurinic endonuclease gene (ARP) fused in frame to the neomycin phosphotransferase II (NPTII) coding region. Exons are represented by gray boxes and introns as lines. Positions of the forward (F1) and reverse (R1 and R2) primers used for the RT-PCR and qRPN12a F and R primers used for qPCR are indicated. (b) Total RNA was extracted, reverse transcribed and used to amplify the RPN12a-NPTII (42 cycles) and wild-type RPN12a transcripts (35 cycles). The primers used for the reaction are indicated. Proteasome β subunit 1 (PBA1) elongation factor 1-α (EF-1-α) are controls. (c) Quantitative real-time RT-PCR analyses of RPN12a and RPT2a levels in C24, rpn12a-1 and sor1 seedlings. The reference gene was ACT2. The transcript levels in C24 were assigned the value of 1. The data represent average relative quantity (RQ) values of three replicates, and the bars denote the RQMin to RQMax. The difference in transcript levels between C24 and the mutants is marked (***, P < 0.0001; ANOVA with Bonferroni multiple comparison test).

Immunoblotting analyses using anti-RPN12a antibodies showed that the sor1 mutant does not accumulate the RPN12a-NPTII fusion protein (Figure 4). The RPN12a abundance in sor1 was increased compared to rpn12a-1 and similar to the wild-type. We were also unable to detect the RPN12a-NPTII fusion in sor1 by using

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anti-NPTII antisera (Figure 4). In the rpn12a-I mutant, a fraction of the assembled 26SP contains the fusion protein leading to a decrease in total cellular 26SP activity and a compensatory increase in the expression of proteasome subunit genes\(^\text{15, 28}\). In the sor1 mutant, with no or little fusion protein, 26SP function is expected to be restored back to the wild-type level. Indeed, immunoblotting analyses with the anti-PBA1 antibodies showed that the abundance of the 20S proteasome subunit PBA1 in sor1 seedlings was comparable to that of the wild-type, indicating that proteasome activity was restored to optimal levels and that feedback up-regulation of proteasome subunit genes was halted (Figure 4).

Taking into account both the result of the Km resistance tests (Figure 2) and the expression data (Figure 3 and Figure 4), we concluded that the sor1 mutation strongly but incompletely suppresses the formation of the RPN12a-NPTII fusion transcript which was sufficient to restore 26SP function back to the wild-type level.

**sor1 weakens the STOP codon-associated 5’ splice site in rpn12a-I**

To find the mutation that causes the sor1 phenotype, we amplified and compared the sequences of the RPN12a-NPTII chimeric gene from sor1 and rpn12a-I. No mutations were found in NPTII, indicating that the loss of Km resistance and NPTII abundance was not caused by any disruption of the NPTII coding region. We also did not detect any changes in the RPN12a coding region, but did find a single nucleotide change immediately downstream of the RPN12a STOP codon (Figure 5). Sequencing of the entire region between RPN12a and NPTII did not reveal any additional mutations, confirming that the RPN12a STOP codon-associated G to A mutation was indeed sor1.

To analyze how this G-to-A substitution leads to reversion of the rpn12a-I phenotype, we manually compared the consensus sequence for 5’ splice sites in *Arabidopsis*\(^\text{19}\) with the sequence of the exon/intron junction that precedes the RPN12a STOP codon in rpn12a-I and sor1 (Figure 6a). The alignment revealed that both the intron and exon residues adjoining the splice junction of the mutants match the consensus well. Interestingly, the sor1 mutation changes a consensus G at the fifth position of the intron into an A, thus weakening the 5’ splice site of the chimeric intron. The G at the position +5 is thought to be required for efficient binding of U1snRNP\(^\text{29}\). Reduced splicing of the chimeric intron between the RPN12a and NPTII coding regions is predicted to lead to a reduced accumulation of the RPN12a-NPTII transcript and protein (Figure 6b and 6c). The combination of reduced intron splicing and unaffected 3’ end processing is therefore predicted to lead to a dramatic shift in favor of the formation of the wild-type RPN12a transcript, and thus to the accumulation of the RPN12a protein back to the wild-type level, which is what we observed in sor1 seedlings.

**Conclusions**

Collectively, the results shown here validate our earlier interpretation of the effects of the rpn12a-I mutation on RPN12a expression and 26SP function\(^\text{15}\). In the original study, we proposed that the partial loss of 26SP function in rpn12a-I seedlings is caused by the competition between RPN12a and RPN12a-NPTII transcript

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**Figure 4. The RPN12a-NPTII fusion protein is absent in the sor1 mutant.** Total protein was isolated from two-week-old wild-type (C24), rpn12a-I and sor1 seedlings and used for immunoblotting analyses with RPN12, NPT and PBA1 antisera. In addition to the RPN12a and RPN12a-NPTII fusion proteins, the anti-RPN12 sera also recognized two proteins (cross) that are not related to RPN12a. Ponceau S‐stained membrane showing the large RuBisCO subunit (LSU) is presented as a loading control. The size of the proteins used as molecular mass standards is shown on the right-hand side.

**Figure 5. Sequence alignment of the RPN12a gene (At1g64520) in rpn12a-I and sor1.** Genomic DNA fragment was amplified using F1 and R2 primers (presented in Figure 3), sequenced and the sequence was aligned using Vector NTI suite. Alignment of the region starting with base pair 1615 and ending with base pair 1804 of the annotated RPN12a gene is presented using BoxShade 3.2. The red arrowhead points to the sor1 mutation and the RPN12a STOP codon is boxed in red.
processing that leads to a decrease of RPN12a protein levels and thus, to a decrease in the abundance of wild-type 26SP particles. Our finding that suppression of RPN12a-NPTII accumulation was sufficient to restore RPN12a accumulation and reverse the plant development and cytokinin sensitivity back to the wild-type level validates the proposed interpretation and accentuates the importance of optimal 26SP abundance for Arabidopsis growth and cytokinin regulation.

**Author contributions**

JK and JAS designed the experiments, performed all experiments except the qPCR analyses, analyzed the data, and wrote the manuscript. YL performed the qPCR analyses and critically revised the second version of the manuscript.

**Competing interests**

No relevant competing interests were disclosed.

**Grant information**

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References


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Version 2

Referee Report 01 July 2013
doi:10.5256/f1000research.1618.r1037

Patrick Masson
Department of Genetics, University of Wisconsin-Madison, Madison, WI, USA

This revised manuscript includes both a better description of the mutagenesis and screening strategy that led to the isolation of sor1 and a new experiment quantifying transcript levels. This addresses two of my initial questions. A transgenic-rescue experiment aimed at verifying that the mutation detected immediately downstream of the RPN12a STOP codon truly corresponds to sor1 has not been carried out. Although I still believe this would be useful to verify this conclusion, I also think that all other data lead us to believe in the proposed model.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 25 June 2013
doi:10.5256/f1000research.1618.r1024

Vitaly Citovsky
Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, NY, USA

The revised paper addresses my suggestion and strengthens the manuscript.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 06 March 2013
doi:10.5256/f1000research.1151.r812
This manuscript nicely documents the molecular basis for an intragenic suppressor of the \textit{rpn12a-1} exon-trap mutation of \textit{Arabidopsis}, which weakens a chimeric 5' splice site that fuses the \textit{RPN12} open reading frame to the \textit{NPTII} coding region in the original mutation. These authors had previously shown that the T-DNA insertion of \textit{rpn12a-1} results in a competition between 3' splicing of \textit{RPN12A} RNA (using a donor splice site that overlaps with the stop codon and the acceptor splice site upstream of its \textit{NPTII} coding region of the T-DNA) and its normal polyadenylation. They had suggested that a fraction of the mutant transcripts encoded a non-functional \textit{RPN12A-NPTII} fusion protein that, upon insertion into the proteasome, altered its activity. Hence, in the original mutant, overall altered proteasome activity resulted in pleiotropic phenotypes associated with cytokinin resistance compared to wild type. In this suppressor line, a point mutation 5 nucleotides within the cryptic intron altered this competing splicing, thereby restoring more efficient polyadenylation and production of enough functional \textit{RPN12a} protein to restore fully functional proteasome activity. Hence, this analysis confirms the initial interpretation of the source of phenotypes associated with \textit{rpn12a-1}, and documents an interesting example of alteration through mutation of a balance between 3' splicing and polyadenylation of a precursor RNA.

The design of this work, protocols and results are well presented and justify the conclusions. However, I had a few minor questions on this work:

- How many suppressors were identified in this analysis? Were other intragenic suppressors identified?

- Considering the information provided here, one would suspect that \textit{sor1} is a dominant mutation. Is it? If it is, has an experiment been carried out to show that a transgenic copy of the suppressed \textit{rpn12a-1} sor rescues the cytokinin-resistance phenotype of \textit{rpn12a-1}?

- Analysis of kanamycin resistance in wild type C24, \textit{rpn2a-1} and \textit{sor1} seedlings showed that \textit{sor1} retains a reasonably high level of resistance compared to wild type (Fig 2). Yet, the molecular characterization described in Figures 3 and 4 shows no evidence of \textit{NPTII} transcript or protein being produced in this suppressor. Is this a problem of experimental sensitivity? A brief discussion of this observation should be included in the text.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

\textbf{Competing Interests:} No competing interests were disclosed.
Vitaly Citovsky
Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, NY, USA

This is a very nicely executed and clearly written work. The results are clear, and they support the authors’ conclusions and previously published hypotheses of proteasome involvement in cytokinin response.

One potential enhancement would be to use qPCR to quantify the amount of transcripts, especially since these data represent one of the major findings of the paper.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.