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Organismal benefits of transcription speed control at gene boundaries

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Abstract

RNA polymerase II (RNAPII) transcription is crucial for gene expression. RNAPII density peaks at gene boundaries, associating these key regions for gene expression control with limited RNAPII movement. The connections between RNAPII transcription speed and gene regulation in multicellular organisms are poorly understood. Here, we directly modulate RNAPII transcription speed by point mutations in the second largest subunit of RNAPII in Arabidopsis thaliana. A RNAPII mutation predicted to decelerate transcription is inviable, while accelerating RNAPII transcription confers phenotypes resembling auto-immunity. Nascent transcription profiling revealed that RNAPII complexes with accelerated transcription clear stalling sites at both gene ends, resulting in read-through transcription. The accelerated transcription mutant NPRB2-Y732F exhibits increased association with 5’ splice site (5’SS) intermediates and enhanced splicing efficiency. Our findings highlight potential advantages of RNAPII stalling through local reduction in transcription speed to optimize gene expression for the development of multicellular organisms.

Keywords NET-seq; speed; splicing; stalling; transcription

Introduction

A decisive step during gene expression is the conversion of the DNA sequences of a gene into pre-mRNA by RNA polymerase II (RNAPII) transcription. Profiles of RNAPII transcription across genes in eukaryotes revealed two main RNAPII localization peaks at gene boundaries, near gene transcription start sites (TSSs) and polyadenylation sites (PASs) [1]. At the 3’ end of genes, RNAPII peaks promote nascent RNA 3’ end processing and transcriptional termination in mammals [2,3]. The function of RNAPII peaks at promoter-proximal regions near TSSs is actively debated. On the one hand, “pause-release” of RNAPII can facilitate rapid induction of gene expression [4]; on the other hand, imaging of Drosophila and human RNAPII at promoter-proximal positions revealed rapid turnover, arguing against stable “pausing” of the same population of RNAPII complexes over time [5,6]. In metazoans, the negative elongation factor (NELF) complex promotes promoter-proximal pausing of RNAPII by limiting RNAPII mobility [7]. However, NELF is conspicuously absent in yeast and plants, which implies that many organisms use alternative mechanisms to stall RNAPII at promoter-proximal region (i.e., RNAPII stalling) [8]. In gene bodies, RNAPII accumulates at exon–intron boundaries and exhibits distinct accumulation profiles for exons with alternative splicing (AS) outcomes [9,10]. The efficiency of splicing may hence be coupled to the local speed of RNAPII elongation at exon–intron boundaries [11]. In summary, peaks of accumulated RNAPII represent sites with reduced RNAPII forward movement, which may facilitate the integration of cellular signals to control gene expression post-initiation by co-transcriptional RNA processing [12].

RNAPII forward movement depends on the dynamics of the trigger loop (TL), a central structure in the RNAPII active center [13–15]. In addition, RNAPII backtracking induced by weak RNA–DNA hybrids (i.e., nucleotide misincorporation) limits RNAPII forward movement [16–18]. A “gating tyrosine” in the RNAPII second largest subunit RPB2 (i.e., Y769 in budding yeast Rpb2)stacks with the first backtracked nucleotide and is proposed to prevent further backtracking [19] and is also positioned to interact with the TL when in its closed, catalysis-promoting state. Point mutations in budding yeast Rpb1 TL residues and Rpb2 TL-interacting residues alter the RNAPII elongation speed in vivo [20–24]. Such “kinetic RNAPII mutants” have informed greatly on the effects of altered transcription speed on gene expression and transcription-related phenotypes. For example, the budding yeast rpb2-P1018S slow transcription mutant (i.e., rpb2-10) promotes RNAPII arrest and reduces transcription processivity [25,26]. Moreover, kinetic RNAPII mutants displaying accelerated transcription favor the use of...
upstream TSSs, while mutants displaying slow transcription tend to use downstream TSSs [27]. Variations of transcription speed alter profiles of co-transcriptional chromatin signatures and of RNAPII C-terminal domain (CTD) phosphorylation that impact pre-mRNA processing [28–30]. These observations indicate a profound effect of RNAPII transcription elongation speed on gene expression. The important question of whether growth and differentiation programs in a multicellular organism can be executed when RNAPII carries kinetic point mutations remains largely unclear.

Here, we altered RNAPII transcription activity in Arabidopsis through point mutations in NRPB2, the second largest subunit of Arabidopsis RNAPII. A mutant accelerating RNAPII transcription triggered phenotypes consistent with auto-immunity, but was able to execute key steps of pattern formation and organogenesis. A mutation predicted to decrease RNAPII transcription speed was inviable. Nascent RNAPII transcription profiling revealed that the mutant accelerating transcription resulted in reduced RNAPII stalling at both gene boundaries. Our findings highlight mechanistic connections between the intrinsic speed of RNAPII and RNAPII stalling at both gene boundaries that coordinate gene expression in the context of a multicellular organism.

Results

Altering transcription activity of RNAPII by targeted mutagenesis of NRPB2

To alter the in vivo RNAPII transcription activity in whole plants, we generated point mutations in Arabidopsis RNAPII. The target residues were identified in Rpb2, the second largest budding yeast RNAPII subunit. The Rpb2 proline 1018 to serine substitution (rpb2-P1018S) represents the classic slow transcription mutant rpb2-10, and the tyrosine 769 to phenylalanine substitution (rpb2-Y769F) represents a mutation which might influence backtracking and TL function (Fig 1A) [19,31,32]. Sequence alignments identified P979S and Y732F in the highly conserved regions of NRPB2, the second largest subunit of Arabidopsis RNAPII as the equivalent positions to budding yeast P1018S (rpb2-10) and Y769F, respectively (Fig 1B). We generated these point mutations in constructs carrying the genomic NRPB2 sequence fused to a C-terminal FLAG-tag driven by the endogenous NRPB2 promoter and integrated them into the nrbp2-2 null mutant background [33] (Fig EV1A). To investigate whether these point mutations affected NRPB2 protein accumulation, we performed Western blotting on FLAG-tagged NRPB2,P979S–FLAG, NRPB2,Y732F–FLAG, and wild-type NRPB2–FLAG (NRPB2WT–FLAG; Fig 1C). We identified several individual transformant lines with comparable steady-state protein levels; thus, any differences we detected in the characterization of these lines would have to be attributed to the effects of the point mutations on RNAPII activity.

The Arabidopsis nrbp2-2 null allele is female gametophytic lethal, but can be transmitted through the male germline with reduced transmission rate [33]. We could hence assay complementation of the gametophytic phenotypes to gain insights into the effects of RNAPII mutants. We assayed the transmission rate of the nrbp2-2 null allele in the plants carrying homozygous NRPB2wt, NRPB2,P979S or NRPB2,Y732F transgenes in nrbp2-2+/− background (Fig EV1A). We would predict increased transmission rate of the nrbp2-2 allele if the gametophytic defects could be complemented. As predicted, NRPB2WT can fully (i.e., to the expected level of 50%) complement the transmission of nrbp2-2 compared to non-transformed controls (Fig 1D). Interestingly, NRPB2,Y732F could almost fully complement nrbp2-2 transmission, while NRPB2,P979S did not significantly increase transmission rate compared to non-transformed controls (Fig 1D). These data suggest that NRPB2,P979S fails to provide the RNAPII activity necessary for germline development. Indeed, silhouette dissection revealed that the germline defects in NRPB2,P979S nrbp2-2+/− were associated with reduced fertility and ovule abortion (Fig EV1B–D). Consistently, we identified plants homozygous for both NRPB2wt transgene and nrbp2-2 mutant (NRPB2,Y732F+/+ nrbp2-2−/−) while NRPB2,P979S+/+ nrbp2-2−/− genotype could not be recovered. Remarkably, when all RNAPII complexes carried the NRPB2,Y732F mutation (i.e., NRPB2,Y732F+/+ nrbp2-2−/−) we observed viable plant growth and development. These plants exhibited a dwarfed stature (Figs 1E and EV1E), but resembled Arabidopsis seedlings concerning basic patterning and organ formation. The dwarfed stature was reminiscent of mutants displaying auto-immunity, which is often associated with increased expression of pathogen-related (PR) genes [34]. Indeed, we detected elevated expression of PRI1, PRI2, and PR5 in NRPB2,Y732F+/+ nrbp2-2−/− compared to NRPB2wt+/+ nrbp2-2−/− (Fig EV1F). These data highlight important roles of the ability to control the speed of RNAPII transcription during plant growth and development. In summary, Arabidopsis RNAPII harboring the NRPB2,P979S point mutation failed to provide viable RNAPII activity during gametogenesis. However, the NRPB2,Y732F mutation can partly rescue the germline defects in nrbp2-2 null mutants and allow plant growth and basic aspects of development.

NRPB2,Y732F accelerates RNAPII transcription in vivo

To investigate the effect of NRPB2,Y732F on RNAPII transcription speed, we first tested whether the equivalent rpb2-Y769F mutant in budding yeast classifies as a fast or slow RNAPII transcription mutant by assaying its sensitivity towards mycophenolic acid (MPA) and Mn2+ [35,36]. Budding yeast RNAPII mutants conferring enhanced catalytic activity (RNAPII fast mutants) are more sensitive towards Mn2+ than the RNAPII slow mutants [20]. In budding yeast, RNAPII fast mutants are sensitive to MPA due to deficient expression of IMD2 gene, which counteracts the inhibition of GTP synthesis by MPA. RNAPII slow mutants tend to be resistant to MPA due to the constitutive IMD2 expression [21]. rpb2-Y769F exhibited strong growth defects towards MPA and Mn2+ while we observed no effect for rpb2-P1018S (Fig EV2A). rpb2-Y769F thus shows a growth phenotype consistent with fast RNAPII transcription mutants [20]. Interestingly, the rpb2-Y769F/P1018S double mutant exhibited mild sensitivity towards MPA compared to either single mutant (Fig EV2A), consistent with a complementary effect on transcription speed as seen across many RNAPII active site mutations in budding yeast [27]. Primer extension analyses of alternative TSSs usage of the ADH1 gene represent an additional assay for RNAPII catalytic rate and therefore putative elongation speed [27], where catalytically hyperactive RNAPII mutants exhibit an upstream shift of TSS. In agreement with previously characterized fast RNAPII transcription mutants, rpb2-Y769F shifts the ADH1 TSS upstream compared to wild type or other Y769 substitutions (Fig EV2B). We
next tested the combinations of rpb2-Y769F with TL residue mutants previously demonstrated to alter RNAPII transcription speed. rpb2-Y769F was synthetically lethal with previously characterized fast RNAPII transcription mutants such as rpb1-L1101S, rpb1-E1103G, and rpb1-G1097D (Fig EV2C), suggesting that these combinations synergistically accelerated RNAPII transcription and supporting the interaction between Y769 and TL residues. Conversely, rpb2-Y769F suppressed the growth defect of previously characterized slow RNAPII transcription mutants such as rpb1-F1086S, rpb1-H11085Q, and rpb1-H11085Y [27,35] (Fig EV2C), suggesting compensatory effects on transcription speed when combining these “slow” mutations with rpb2-Y769F. In conclusion, our results characterized budding yeast rpb2-Y769F as a mutation conferring phenotypes consistent with hyperactive RNAPII mutants which increase RNAPII transcription speed.

To investigate the in vivo RNAPII transcription speed of Arabidopsis RNAPII carrying the NRPB2Y732F mutation, we developed an assay to monitor nascent RNAPII elongation after rapid transcription induction. To avoid time-consuming sample handling and processing issues associated with RNAPII chromatin

**Figure 1.** Altering transcription activity of RNAPII by targeted mutagenesis in NRPB2.

A Schematic drawing of *Saccharomyces cerevisiae* RNAPII transcription active center: Trigger loop is shown in blue. TL-interacting Rpb2 domain is shown in beige. Proline 1018 (P1018, green) and gating tyrosine 769 (Y769, red) are highlighted. The schematic drawing is based on PDB: 2ezh [15].

B Protein sequence alignment of RNAPII Rpb2 Y769 and P1018 regions in *S. cerevisiae* and Arabidopsis thaliana. P979S and Y732F are the yeast equivalent point mutations in Arabidopsis. The color scheme indicates conservation from variable (blue) to conserved (red).

C Detection of NRPB2WT-FLAG, NRPB2P979S-FLAG, and NRPB2Y732F-FLAG protein by Western blotting in NRPB2WT+/-, NRPB2P979S+/-, and NRPB2Y732F+/- respectively. Histone H3 was used as an internal control, and total protein level detected by stain-free blot was used as a loading control. Quantification was done by normalizing to the loading control and anti-H3 blot based on three independent replicates.

D Transmission rate of nrbp2-2 allele in nrbp2-2+/− line (n = 197) and nrbp2-2+/- lines combined with homozygous NRPB2P979S-FLAG+/− (n = 280), NRPB2Y732F-FLAG+/− (n = 240), and NRPB2P97S-FLAG−/− (n = 210), respectively. Fisher’s exact test was used as a statistical test, three asterisks denote P < 0.001 between samples, and n.s stands for not significant.

E Image of homozygous mutant nrbp2-2 fully complemented by NRPB2P979S-FLAG (top, NRPB2P979S+/- nrbp2-2+/−) and partially complemented by NRPB2Y732F-FLAG (bottom, NRPB2Y732F+/- nrbp2-2+/−). Plants were grown for 4 weeks in soil. Scale bars represent 1 cm.

Source data are available online for this figure.
immunoprecipitation from plants (RNAPII-ChIP) [25,37], we analyzed nascent RNA attached to RNAPII to monitor RNAPII elongation [38]. We identified three pathogen resistance-related Toll/interleukin receptor (TIR)-type NB-LRR genes AT4G19520, AT5G41740, and AT5G41750 [39,40] that are rapidly induced by flagellin 22 treatment. To monitor the “waves” of RNAPII elongation on these three genes after transcriptional induction, we performed a time course experiment during flagellin 22 treatment and determined the RNAPII signal by analyzing nascent RNA attached to RNAPII [38]. We chose NRBP2WT-FLAG/+/+ Col-0 and NRBP2Y732F-FLAG/+/+ Col-0 as material for this assay since we detected no differences in growth and immune response in this background. In brief, FLAG-tagged NRBP2WT and NRBP2Y732F proteins were immunoprecipitated by anti-FLAG antibody; RNAPII-associated RNA was purified and used in RT-qPCR analyses of three locations spanning these genes (Fig 2A). When gene induction is well synchronized, fast transcription is expected to show higher nascent RNA level in the gene body and towards the 3’ end of candidate genes during flagellin 22 treatment. We found that the candidate genes were rapidly induced by flagellin 22 treatment, as we detected an increase in nascent RNA level at probe 1 of these genes from 0 to 4 min after treatment (Figs 2B and C, and EV2E). Furthermore, data for the probe capturing RNAPII transcription shortly after induction (i.e., probe 1) suggest that these genes were induced with similar kinetics and to similar levels in NRBP2WT and NRBP2Y732F. Interestingly, we found that NRBP2Y732F showed higher nascent RNA level than NRBP2WT at probe 2 and probe 3 located further into the gene, from 3 min of flagellin 22 treatment onwards (Figs 2B and C, and EV2E). These data suggest that although wild-type RNAPII and mutant RNAPII were equally induced near the 5’ ends of genes, the NRBP2Y732F RNAPII reaches the 3’ ends of genes earlier than NRBP2WT, supporting faster RNAPII transcription in the NRBP2Y732F mutants. In summary, we detect evidence that the Arabidopsis NRBP2Y732F mutant exhibits accelerated RNAPII transcription in vivo.

**Accelerated RNAPII transcription reduces promoter-proximal RNAPII stalling**

To study the genome-wide effects of accelerated RNAPII transcription speed in NRBP2Y732F, we performed plant Native Elongating Transcript sequencing (plaNET-seq) to monitor nascent RNAPII transcription [8]. Two independent replicates of plaNET-seq were performed for NRBP2Y732F/+ nrpb2-2/+ mutant and NRBP2WT/+ nrpb2-2/+ control (Fig EV3A and B). Nascent RNA profiling in Arabidopsis revealed RNAPII stalling peaks near the beginning of transcription units in promoter-proximal regions. The positioning of the first nucleosome correlates well with the position of promoter-proximal RNAPII stalling in Arabidopsis [8]. To address the role of transcription speed in regulating promoter-proximal stalling, we investigated the RNAPII signal in promoter-proximal regions from plaNET-seq in NRBP2Y732F/+ nrpb2-2/+ and NRBP2WT/+ nrpb2-2/+ (Fig 3A). A metagene plot showing plaNET-seq RNAPII signal in a 1 kb region centered at the +1 nucleosomes [41] revealed that NRBP2Y732F reduced promoter-proximal RNAPII stalling centered at the +1 nucleosome position genome-wide compared to NRBP2WT (Fig 3B). The metagene-level reduction in RNAPII stalling in NRBP2Y732F was confirmed when the plaNET-seq signal was anchored at TSSs (Fig EV3C). To further quantify this effect, we calculated the RNAPII stalling index for well-expressed genes (plaNET-seq signal FPKM > 10, n = 6,596), which represents relative enrichment of RNAPII signal at promoter-proximal regions compared to the whole gene body. This analysis quantified a 35% reduction in the median value of RNAPII promoter-proximal stalling index in NRBP2Y732F compared to NRBP2WT (Fig 3C). These data illustrate that a restriction of RNAPII transcription speed contributes strongly to the formation of characteristic promoter-proximal RNAPII peaks.

**Accelerated transcription increases nascent RNAPII signal in gene bodies**

We observed increased RNAPII signals in NRBP2Y732F compared to NRBP2WT at intragenic positions downstream of promoter-proximal stalling sites (Fig EV3D). A metagene analysis of RNAPII activity across gene bodies confirmed this observation on a genome-wide scale (Fig 3D). Increased RNAPII signal in gene bodies could be reconciled by less RNAPII at promoter-proximal stalling regions in NRBP2Y732F compared to NRBP2WT. Consistently, increased nascent transcription in gene bodies in NRBP2Y732F correlated with increased plaNET-seq metagene profiles of exons and introns (Fig 3E and F). Interestingly, we detected an accumulation of...
exonic plaNET-seq signal towards the 3' end of exons in NRPB2_{Y732F} (Fig 3E). This effect was insensitive to the exon length (Fig EV3E–G). Exon–intron boundaries may thus trigger a pile-up of nascent RNAPII transcription when transcription is accelerated. In introns, accelerated RNAPII transcription amplifies nascent RNAPII signal compared to NRPB2_{WT} and resulted in a uniform accumulation profile, which can be observed in metagene plots for introns of variable length genome-wide (Figs 3F, and EV3H–J). We next tested possible connections between increased intragenic nascent RNAPII signal and splicing regulation. However, the fast mutant showed increased signal over both constitutive and alternative exons and introns (Fig EV3K–N). In conclusion, accelerated RNAPII transcription in NRPB2_{Y732F} resulted in increased nascent RNAPII transcription in gene bodies.
Accelerated transcription enhances intron splicing and exon skipping

plaNET-seq co-purifies splicing intermediates due to co-transcriptional spliceosome association with RNAPII (Fig 4A). The splicing intermediates appear as single-nucleotide sharp peaks at 5ʹ splicing site (5ʹSS) and 3ʹ splicing site (3ʹSS) and thus can be distinguished from the nascent reads [10,42]. We detected an increased fraction of splicing intermediate reads corresponding to 5ʹSS in plaNET-seq of NRPB2WT compared to NRPB2WT, while no obvious difference could be detected for 3ʹ splicing intermediates (Fig 4B). These data suggested an increased association of accelerated RNAPII transcription with splicing intermediates overlapping a 5ʹSS. Since 5ʹ splicing intermediates are associated with the spliceosome, we predicted that higher RNAPII coverage in gene bodies could increase spliceosome association and perhaps enhance splicing in NRPB2WT. To test this idea, two independent replicates of RNA-seq were performed for NRPB2WT+/- and NRPB2WT+/- seedlings (Fig EV4A). RNA-seq detects predominantly spliced transcripts with a characteristic signal intensity profile matching annotated exons. However, we noticed RNA-seq signal corresponding to some introns, presumably representing regulatory or poorly spliced introns (i.e., retained introns). Interestingly, initial visual

Figure 3. Accelerated RNAPII transcription reduces promoter-proximal RNAPII stalling and enhances RNAPII activity in gene body.

A plaNET-seq signal of RNAPII in the promoter-proximal region of AT1G70600 in NRPB2WT+/- nrbp2-2/- (NRPB2WT, blue) and NRPB2WT+/- nrbp2-2/- (NRPB2WT, red). Arrows indicate the RNAPII signal at the region of promoter-proximal stalling.

B Metagene profile of plaNET-seq mean signal of RNAPII in a 1 kb window centered at the +1 nucleosome in Arabidopsis genes (n = 25,474) in NRPB2WT+/- nrbp2-2/- (NRPB2WT, blue) and NRPB2WT+/- nrbp2-2/- (NRPB2WT, red). The significance of differences of plaNET-seq signal in the region from -25 bins to +25 bins around +1 nucleosome between NRPB2WT and NRPB2WT was assessed by a two-sided Mann-Whitney U-test, P = 5.20e-10.

C RNAPII stalling index calculated for all the genes with plaNET-seq FPKM ≥ 10 in NRPB2WT, blue) and NRPB2WT, red. Medians of the stalling index are 1.891 and 1.222 for NRPB2WT and NRPB2WT, respectively. *** denotes P-value = 0.001 by Wilcoxon signed-rank test. The solid horizontal lines and box limits represent median, lower and upper quartiles of data values in each group. The upper and lower whiskers extend to the largest or smallest value, respectively, no further than 1.5*IQR from the relevant quartile.

D Metagene profile of plaNET-seq mean signal over whole genes (length from 0.5 kb to 5 kb, scaled to 500 bins, n = 27,042) in NRPB2WT+/- nrbp2-2/- (NRPB2WT, blue) and NRPB2WT+/- nrbp2-2/- (NRPB2WT, red).

E Metagene profile of plaNET-seq mean signal of RNAPII in exons (length from 50 to 300 bp, scaled to 100 bins, n = 73,925) in NRPB2WT+/- nrbp2-2/- (NRPB2WT, blue) and NRPB2WT+/- nrbp2-2/- (NRPB2WT, red). Pink dashed line rectangle illustrates the amplitude of differences between the minimum and the maximum of RNAPII signal across the exons. A two-sided Mann-Whitney U-test was used to assess the plaNET-seq signal of NRPB2WT (blue) and NRPB2WT (red) in exons, P < 1e-16.

F Metagene profile of plaNET-seq mean signal of RNAPII in introns (length from 50–300 bp, scaled to 100 bins, n = 102,260) in NRPB2WT+/- nrbp2-2/- (NRPB2WT, blue) and NRPB2WT+/- nrbp2-2/- (NRPB2WT, red). A two-sided Mann-Whitney U-test was used to assess the plaNET-seq signal of NRPB2WT (blue) and NRPB2WT (red) in introns, P < 1e-16.
inspection of several retained introns indicated that accelerated RNAPII transcription in NRPB2Y732F appeared to decrease intronic RNA-seq signal (Fig 4C). Strikingly, this finding is supported by a genome-wide decrease in the fraction of intronic RNA-seq signal across all genes (Fig 4D), suggesting a genome-wide trend of increased splicing efficiency in plants when RNAPII transcription is accelerated. A systematic genome-wide analysis identified 1,517 differentially expressed (DE) introns from the RNA-seq data of NRPB2Y732F compared to NRPB2WT. The majority (1,334 out of 1,517) of DE introns exhibit decreased fraction of intronic reads (Table EV2). We identified similar numbers of DE exons with increased or decreased expression in NRPB2Y732F compared to NRPB2WT, while we detected many more introns with decreased expression (Fig 4E). Quantification of DE exons revealed a small yet significant reduction in expression (Fig 4F) that we visualized for internal exons of the AT1G58060 and AT3G05680 genes (Fig 4G). In contrast, we detected a stronger decrease for DE introns in NRPB2Y732F mutant compared to NRPB2WT (Fig 4H).

We next tested alternative 5'SS and 3'SS usage (Fig EV4B) in the NRPB2Y732F mutant compared to NRPB2WT and found a trend to shift 5'SS upstream and 3'SS downstream (Fig EV4C–E). We note that a downstream shift of 3'SS is consistent with effects observed...
in the splicing factor mutant ntr1 linked to increased transcription speed in Arabidopsis [16]. In summary, our RNA-seq data revealed multiple effects of accelerated RNAPII transcription on splicing in Arabidopsis. Our analyses highlighted reduced intron retention as the most notable effect of altered RNAPII activity in Arabidopsis on splicing. The data support the idea that inefficient splicing of these introns in wild type may be interpreted through a model where RNAPII transcription speed could be limiting their splicing.

**Accelerated RNAPII transcription reduces RNAPII stalling at gene ends**

pLaNET-seq resolves peaks of RNAPII activity at 3′ ends of plant genes. This localized reduction in transcription speed at gene ends may assist RNAPII transcriptional termination. To test this hypothesis, we investigated the RNAPII stalling peaks at 3′ ends of genes by pLaNET-seq in the fast transcription mutant NRPB2Y732F compared to NRPB2WT. We detected RNAPII stalling downstream of poly(A) sites (PASs) of Arabidopsis genes in NRPB2WT (Fig 5A). In contrast, PAS-stalling peaks of RNAPII in this region were often undetectable in NRPB2Y732F, as shown for the AT2G21410 gene (Fig 5A). A metagene analysis confirmed RNAPII peaks downstream of PAS at 3′ gene ends in NRPB2WT and a strong reduction in NRPB2Y732F genome-wide (Fig 5B). These data connect increased RNAPII transcription speed and reduced RNAPII stalling at gene ends downstream of PAS. If RNAPII stalling were promoting transcriptional termination, we would expect termination defects in NRPB2Y732F. Indeed, genome browser screenshots indicated higher RNAPII signal downstream of the PAS-stalling region in NRPB2Y732F in comparison with NRPB2WT (Fig 5A), suggesting transcriptional read-through as a consequence of increased transcription speed. To quantify this effect genome-wide, we determined the transcriptional read-through lengths in NRPB2WT and NRPB2Y732F. We used a statistical model which was based on empirical distributions of pLaNET-seq tag counts in both genic and intergenic regions (see Materials and Methods). Strikingly, we observed that NRPB2Y732F extended transcriptional read-through genome-wide (FPKM > 5, n = 9,316; Fig 5C). We detected a 115 nt increase of median transcriptional read-through length in NRPB2Y732F compared to NRPB2WT (NRPB2Y732F, 649 nt; NRPB2WT, 534 nt; Fig 5D). NRPB2Y732F accelerating transcription speed thus reduces RNAPII termination efficiency and extends transcriptional read-through. The process of RNAPII termination is sensitive to the RNAPII active site and putative catalysis, consistent with a model where increased RNAPII speed alters kinetic competition between transcriptional stalling, termination, and further elongation.

Transcriptional read-through blurs the boundaries of transcription units, which could result in overlapping transcripts and potential gene expression conflicts. To investigate this, we focused on read-through transcription of tandemly oriented genes, where transcription read-through from upstream-located genes may invade downstream genes. RNAPII with accelerated transcription speed is expected to extend transcriptional read-through into the intergenic space (i.e., gaps) between the PAS of an upstream gene and the TSS of a downstream gene (PAS-TSS gaps). Indeed, NRPB2Y732F shows higher RNAPII signal than NRPB2WT in the second half of PAS-TSS gaps (n = 5,753) while RNAPII in NRPB2WT stalls downstream of PAS in the first half of PAS-TSS gaps (Figs 5E and EV5A). We further investigated pLaNET-seq RNAPII signal in PAS-PAS gaps of paired genes facing each other in “tail-to-tail” orientation (n = 1,384). Also for this subset of genes, NRPB2Y732F lacked the characteristic RNAPII PAS-stalling in the first half of PAS-PAS gaps and showed significantly higher RNAPII signal in the second half of PAS-PAS gaps (Figs SF and EV5B). These data suggest that accelerated transcription speed triggers transcriptional read-through genome-wide, resulting in overlapping transcripts and potential gene expression conflicts. In conclusion, our data highlight connections between reduced speed of RNAPII transcription at gene ends (i.e., PAS-stalling) and the termination of RNAPII transcription, linking the speed of transcription to spatial separation of plant transcription units.
Our findings highlight molecular and organismal consequences of altered RNAPII elongation speed in a multicellular organism. The two main peaks of RNAPII localization in genomes at gene boundaries were depleted when transcription speed was accelerated (Fig 6A and B). Accelerated RNAPII transcription impacted gene expression after transcriptional initiation, through profound effects on splicing and transcriptional termination. Our data support that transcription speed control at gene boundaries is a key step in gene expression of multicellular organisms.

**Discussion**

RNAPII transcription speed and organismal development

While we succeeded in generation of viable plants carrying a fast RNAPII mutation, we were unable to obtain plants with a mutation in a conserved residue that reduced RNAPII transcription speed in yeast. This observation is reminiscent of embryonic lethality in mice through a point mutation in the largest RNAPII subunit that
Promoter-proximal stalling represents a common feature of transcription throughout eukaryotic genomes [46]. The purpose of promoter-proximal RNAPII stalling is debated actively, yet a reduction in RNAPII transcription speed during stalling could be part of a checkpoint regulating gene expression. In organisms without NELF, for example, plants, promoter-proximal RNAPII stalling correlates with the position of the +1 nucleosome encountered by the transcription machinery [8]. Accelerated transcription in NRPB2Y732F decreased promoter-proximal stalling and resulted in increased intragenic RNAPII transcription. Our data thus suggest that a reduction in RNAPII elongation speed near promoters facilitates the accumulation of promoter-proximal RNAPII peaks. In Arabidopsis, these peaks form independently of NELF and perhaps form through contributions by nucleosome barriers that correlate well with the peak position [8,47].

Possible advantages of promoter-proximal RNAPII stalling include a reduced response time to adjust gene expression to new environmental conditions. Instead of initiating the process of RNAPII transcription from recruitment and complex assembly, stalled RNAPII may represent pre-assembled and elongation competent RNAPII complexes waiting for signals to transcribe the full gene [1]. Defense signaling is crucial for plant fitness and regulated with fast temporal dynamics, perhaps achieved by a release of RNAPII into elongation from promoter-proximal stalling sites. If true, this would predict constitutive defense signaling when transcription is accelerated. Interestingly, NRPB2Y732F/+ nrpb2-2/- plants resembled mutants with constitutively active defense signaling [34]. PR gene induction represents a diagnostic molecular hallmark of elevated defense signaling [48]. While alternative molecular explanations for stunted growth in NRPB2Y732F/+ nrpb2-2/- may exist, for example, indirect effects, the induction of PR gene expression is consistent with an auto-immunity phenotype triggered by accelerated transcription. Our data thus provide a potential connection between plant defense signaling, promoter-proximal RNAPII stalling, and the speed of RNAPII transcription. In conclusion, these data imply that transcription speed limits at gene boundaries may benefit plants by avoiding constitutive defense signaling that triggers auto-immunity.

Accelerated RNAPII transcription and RNA processing

Our targeted introduction of candidate point mutations represents a direct approach to address mechanistic links between the speed of RNAPII transcription and RNA processing. Nevertheless, some molecular effects we reported could represent indirect effects caused by differences in growth and development between NRPB2Y732F/+ nrpb2-2/- and NRPB2WT/+ nrpb2-2/-. RNA-seq revealed that intron retention is reduced when RNAPII is accelerated; in other words, splicing efficiency of poorly spliced introns is increased. plaNET-seq data indicate that increased splicing efficiency is associated with the capture of splicing intermediates with 3' terminal bases overlapping 5'SS, perhaps indicating that splicing of retained introns could be increased by promoting RNAPII binding to 5'SS. In conclusion, the speed of RNAPII transcription contributes to plant gene expression by modulating splicing efficiency, particularly at retained introns.

plaNET-seq data informed on transcriptional termination of RNAPII. Strikingly, we found a reduction in RNAPII peaks associated with termination when transcription is accelerated, and an increased distance of read-through transcription downstream of the PAS (Fig 5D). Read-through transcription triggered by elevated temperature has been reported in budding yeast and mammalian cell culture [37,49]. Extended read-through as observed in an accelerated RNAPII transcription mutant may have functional consequences on gene expression. The increased transcriptional read-through may result in gene expression defects for neighboring genes, for example, by transcriptional interference [50,51]. In summary, our data support the idea that a reduction in RNAPII transcription speed promotes RNAPII density peaks in genomes with functional consequences for the process of transcriptional termination.
Materials and Methods

Reagents and Tools Table

<table>
<thead>
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<th>Identifier</th>
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</table>

Methods and Protocols

**Plant material and growth conditions**

The *Arabidopsis* mutant lines generated in this study were based on *A. thaliana* Columbia ecotype (Col-0) background. Generation of transgenic *Arabidopsis* plants was performed by *Agrobacterium*-mediated transformation as described [52]. NRPB2_Y732F and NRPB2_Y732F transgenes were first introduced to Col-0 and then crossed with 

\[
\text{nrpb2-2}^+ \text{/nrpb2-2}^- \text{/nrpb2-2}^+ \text{ and } \text{nrpb2-2}^+ \text{/nrpb2-2}^- \text{ double mutants were screened by genotyping from F3 generation (Fig EV1A).}
\]

For *in vitro* growth, *Arabidopsis* seeds were surface-sterilized and placed on ½ MS media agar plates (1% sucrose) for 12 days, and seedlings were transferred into ½ MS liquid media (1% sucrose) and grown in flasks under 22°C with 16/8-h light/dark and 150 rpm shaking for 2 days. For growth on soil, *Arabidopsis* seeds were sowed on soil directly and undergo stratification in 4°C for 3 days before growth under 22°C with 16/8-h light/dark on soil.

**Plasmid construction**

The construction of vectors for *Agrobacterium*-mediated stable transformation was based on pEarleyGate 302 vector (pEG302). pEG302-NRPB2_Y732F FLAG construct was kindly provided by Craig Pikaard [33]. To generate pEG302-NRPB2_Y732F FLAG and pEG302-NRPB2_Y732F FLAG, pEG302-NRPB2_Y732F FLAG construct was linearized by DraII digestion and used as backbone in
isothermal assembly reactions; the inserts in isothermal assembly are partial genomic NRPB2 sequences containing \( Y732F \) (TAT to TTT) point mutation and P979S (CCG to TCG), respectively. Fragment containing DNA mutation for \( Y732F \) mutant was generated by overlapping PCR (primer pair 3089/3082) fusing two fragments generated by primer pair 3089/3467 and 3082/3466. By using similar strategy, fragment containing DNA mutation for P979S mutant was also generated by overlapping PCR (primer pair 3089/3082) fusing two fragments generated by primer pair 3089/3084 and 3082/3083. Isothermal assembly was performed subsequently to generate pEG302-AtNRPB2\(_{Y732F}\)-FLAG and pEG302-AtNRPB2\(_{Y732F}\)-3082/3083. Isothermal assembly reactions; the inserts in isothermal assembly with gentle rotation (final concentration 0.25 M sucrose, 1.25% Ficoll, 2.5% Dextran T40, 20 mM Tris–HCl pH 7.5, 10 mM MgCl\(_2\), 0.5% Triton X-100, 5 mM DTT, 1× EDTA-free Complete protease inhibitor (Roche)). The nuclear fraction was digested by 600 U DNase I in 0.5 ml Lysis bufer [0.3 M NaCl, 20 mM Tris–HCl pH 7.5, 5 mM MgCl\(_2\), 5 mM β-mercaptoethanol, 1× EDTA-free Complete protease inhibitor (Roche), 0.5% Tween-20, 10 μl RNase inhibitor {moloX GmbH, www.molox.de}] at 4°C for 20 min with shaking at 2,000 rpm on an Eppendorf ThermoMixer. The supernatant of a centrifugation (10,000 g for 10 min at 4°C) was recovered into a new tube and combined with Dynabeads M-270 (Invitrogen) coupled with anti-FLAG antibody (Sigma) for 2 h at 4°C with gentle rotation. Anti-FLAG antibody was coupled with Dynabeads according to the manufacturer’s instructions. After FLAG-IP, beads were washed 6 times using 0.5 ml Wash buffer [0.3 M NaCl, 20 mM Tris–HCl pH 7.5, 5 mM MgCl\(_2\), 5 mM β-mercaptoethanol, 1× EDTA-free Complete protease inhibitor (Roche), and RNase inhibitor]. Bead-bound protein was eluted with 0.5 mg/ml 3xFLAG peptide (ApexBio) for 30 min twice at 4°C. RNA attached to immunoprecipitated proteins was isolated using QIAGEN miRNeasy Mini Kit according to manufacturer’s instructions. Western blot has been done as previously described [38] for input, unbound, and eluted fractions to monitor IP efficiency.

**Nascent RNA isolation**

Isolated nascent RNA was treated with Turbo DNase to remove DNA contamination following the manufacturer’s instruction (Ambion). Hundred nanograms of DNase-treated RNA was used for reverse transcription into cDNA by gene-specific primers following the manufacturer’s instruction of Superscript IV (Invitrogen) kit. Quantitative analysis of the generated cDNA was carried out by qPCR using the GoTaq qPCR Master Mix (Promega) and CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Negative controls lacking the reverse transcriptase enzyme (−RT) were performed alongside all RT–qPCR experiments. qPCR expression level of each primer pair was calculated relative to the level of reference gene \( ACT2 \). All the primers used in RT and qPCR were summarized in Table EV1.

**PlaNET-seq library construction and sequencing**

Libraries for plaNET-seq were prepared as previously described [8]. Nascent RNA from \( \text{NRPB2}_{WT}^{+/+} \text{nrpb2-2}^{−/−} \) and \( \text{NRPB2}_{Y732F}^{+/+} \text{nrpb2-2}^{−/−} \) seedlings was used in plaNET-seq. In specific, the plaNET-seq libraries were constructed according to BioScientific’s NEXTflex Small RNA-Seq Kit v3 using a customized protocol. Two independent replicate libraries were constructed for each plant genotype. Approximately 100 ng RNA was used for each replicate.
The isolated RNA was ligated with 3' linker and fragmented in alkaline solution (100 mM NaCO3 pH 9.2, 2 mM EDTA). The fragmented RNA was cleaned up and subjected to T4 PNK treatment (20 U PNK, NEB) for 20 min at 37°C followed by re-annealing of RT primer (5'-GGCTTGGCACCAGAATTCCA-3'; 70°C, 5 min; 37°C, 30 min; and 25°C, 15 min). The RNA was then re-introduced to the manufacturer's protocol at the adapter inactivation step. The final libraries were quantified with DNA High Sensitivity Kit on Agilent Bioanalyzer 2100 and then sequenced on the Illumina HiSeq 4000 platform in PE150 mode at Novogene (https://en.novogene.com).

**Total RNA extraction and RNA-seq**

Total RNA was extracted from 2-week-old NRPB2WT+/+ nrbp2-2/– and NRPB2Y32F+/+ nrbp2-2/– Arabidopsis seedlings using Plant RNeasy Mini Kit following manufacturing instructions (QiAGEN). Turbo DNase (Ambion) was used to treat extracted RNA using oligo-dT primers and Superscript IV (Invitrogen) as per manufacturer’s instructions. The poly(A)-enriched libraries for RNA-seq were constructed using Illumina TruSeq Sample Prep Kit v2 following the manufacturer’s protocol and quantified on Agilent Bioanalyzer. The sequencing was performed on Illumina HiSeq 4000 platform in PE100 mode.

**Bioinformatics**

All the supporting code for bioinformatics analysis is available at https://github.com/Maxim-Ivanov/Leng_et_al._2019. Alignment and post-processing of plaNET-seq reads were done as previously described [8]. The first 4 bases of both R1 and R2 reads in plaNET-seq are unique molecular identifiers (UMIs). They were trimmed from read sequences and appended to read names using UMI-Tools v0.5.3. After UMI trimming, the 5’-terminal base of R2 corresponds to the 3’-end of original RNA molecule and thus denoted the genomic position of RNAPII active center. R2 reads were aligned to TAIR10 genome assembly using STAR v2.5.2b in transcriptome-guided mode with the following settings: –outSAMmultNmax 1 –alignEndsType Extend5p0Read1 -clip3pAdapterSeq GATCGTCGGACT. Ensembl Plants release 28 was used as the source of transcript annotation for alignment. The BAM files were sorted using Samtools v1.3.1. The following categories of reads were filtered out: (i) PCR duplicates (UMI-Tools); (ii) reads aligned within 100 bp from any rRNA, tRNA, snRNA, or snoRNA gene from Araport11 on either strand (BEDTools v2.23.0); and (iii) reads aligned with MAPQ < 10 (Samtools). The filtered BAM files were imported into R environment v3.5.1 using GenomicAlignments_1.18.1 library. The strand orientation of reads was flipped to restore strandedness of the original RNA molecules. 3’-terminal bases of flipped reads were found to overlap with donor or acceptor splice sites much more frequently than could be expected by chance. Such reads most likely represent splicing intermediates due to co-immunoprecipitation of the spliceosome together with FLAG-tagged RNAPII complexes. These reads were filtered out by overlap with the union of splice sites obtained from both Ensembl Plants 28 (TxDb.Athaliana.BioMart.plantsmart28 package) and Araport11 annotations. In addition, all split reads were removed as possible mature RNA contaminations. The remaining reads are expected to represent the nascent RNA population. Their genomic coverage was exported as strand-specific BigWig and bedgraph files using rtracklayer_1.42.2. For details on the alignment procedure, see 01-Alignment_of_plaNET-Seq_data.sh and 02-Postprocessing_of-_ plaNET-Seq_data.R.

Araport11 annotation was used throughout all further steps of data analysis because it is more comprehensive in terms of non-coding transcripts than both TAIR10 and Ensembl Plants 28 annotations. We adjusted gene borders from Araport11 using coordinates of TSS and PAS tag clusters which were called using CAGEfightR package [53] from the available TSS-seq [50] and direct RNA-seq datasets [54,55], respectively. If multiple TSS or PAS tag clusters were connected to the same gene, the strongest of them was chosen as the new border. For details, see 03_Adjustment_of_Araport11_- gene_boundaries.

To draw metagene plots of plaNET-seq, we merged Bedgraph tracks of the two biological replicates of each genotype. The merged tracks were then normalized to 1 million reads in nuclear protein-coding genes. The X axes of metagene plots represent the genomic intervals of choice (whole genes, exons, introns, etc.) which were scaled to the defined number of bins. Intervals overlapping multiple annotated transcription units were excluded from consideration. Both introns and exons were trimmed by 5 bp each side prior to scaling to avoid possible artifacts. The Y axes show the sequencing coverage averaged between the genomic intervals. The shaded area in metagene plots represents normal-based standard error of mean of normalized plaNET-seq signal at each genomic bin. For details, see 08-Metagene_plots.R. The positions of nucleosomes in Arabidopsis were obtained from the PlantDHS database [56].

To calculate the read-through (RT) length, we considered transcribed genes (plaNET-seq FPKM in WT samples above 5). Genomic intervals for RT length estimation were defined from PAS of the analyzed gene to the nearest downstream TSS. Coordinates of TSS and PAS clusters were called from TSS-seq and direct RNA-seq datasets as described above. For each gene of interest, the empirical distribution of plaNET-seq tag counts in 100-bp sliding window was obtained (the “transcription” model). The “random” model corresponding to the un-transcribed state was represented by Poisson distribution where the rate parameter was estimated from plaNET-seq tag counts in intergenic regions. Then, plaNET-seq tags were counted in every 100-bp window moving in 10-bp steps along the candidate RT genomic window. For each window, the probability to observe at most this tag count under the gene-specific “transcription” model was divided by the probability to observe at least this tag count under the alternative “random” model. The start position of the first window where the probability ratio dropped below 1 was considered as the end of the read-through region. For details, see 04-Read-through_distance.R.

To calculate promoter-proximal RNAPII stalling index for each gene, we first found 100-bp windows with the highest plaNET-seq coverage within the interval (TSS – 100 bp, TSS + 300 bp). Center of this window was considered as the summit of promoter-proximal RNAPII peak. The stalling index was then calculated as the ratio of plaNET-seq coverage in this window vs the whole gene (normalized by gene length). To avoid statistical artifacts, genes shorter than 1 kb or having plaNET-seq FPKM below 1 were skipped from consideration. For details, see 05-Promoter-proximal_stalling_index.R.

RNA-seq reads were adapter- and quality-trimmed by TrimGalore v0.4.3 in paired-end mode and then aligned to TAIR10 by STAR v2.5.2b in local mode. Aligned reads with MAPQ below 10 were
removed by Samtools v1.3.1. BAM files were converted to unstranded Bedgraph and BigWig files using BEDTools genomcov v2.26.0 and kentUtils bedGraphToBigWig v4, respectively. The code was detailed in the section 06-Alignment_of_RNA-Seq_data.sh in the mentioned GitHub page.

Differentially expressed genes were called from RNA-seq data using DESeq2 [57]. Differentially expressed exons and introns were detected independently from the changes in gene expression level by DEXSeq [58]. Exons and introns were defined as disjoint exonic or intronic intervals, respectively, in Araport11. For details, see 07-Differential_expression.R.

To detect the differential usage of alternative 5' and 3' splice sites, transcript isoforms were first quantified by Cufflinks [59]. Then, the Cufflinks output was used to quantify the different AS events extracted from an Arabidopsis reference transcript dataset AtRTD2 [60] with SUPPA2 [61]. For details, see 10-SUPPA2_pipeline.sh and 11-Differential_AS.R scripts.

Data availability

The raw and processed plaNET-seq and RNA-seq data were deposited in Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE133143.

Expanded View for this article is available online.

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Author contributions

Conceptualization: XL, CDK, SM; Methodology: MI, PK, SM; Investigation: XL, PK, IM, MI, SM; Formal analysis: MI, AT, AS; Data curation: MI; Writing—original draft: XL, SM; Writing—review and editing: XL, MI, CDK, PK, PB, AS, SM; Visualization, XL, MI; Resources: CDK, SM; Supervision: CDK, SM; Funding acquisition: SM.

Conflict of interest

The authors declare that they have no conflict of interest.

References

phenotypic landscape of the RNA polymerase II trigger loop. *PloS Genet* 12: e1006321


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