**NTR1 is required for transcription elongation check-points at alternative exons in *Arabidopsis***

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**The interconnection between transcription and splicing is a subject of intense study. We have identified *Arabidopsis* homologue of NTR1 (AtNTR1), a conserved spliceosomal disassembly factor, as protein required for co-transcriptional pausing at alternative splice sites. We report that AtNTR1 is required for the correct expression and splicing of *DOG1*, a regulator of seed dormancy. The *atntr1* mutantsplicing analysis revealed a bias for downstream 5’ and 3’ splice site selection and enhanced exon skipping. Localised decrease in PolII occupancy in *atntr1* at the misspliced exons and introns suggest that this directionality in splice site selection is a manifestation of fast PolII elongation kinetics. In addition, AtNTR1 shows co-localisation with actively transcribing PolII and can be detected by ChIP at target genes. Minigene analysis confirmed that strong alternative splice sites constitute a NTR1-dependent transcriptional road block. Plants with compromised PolII endonucleolytic cleavage showed opposite splicing and PolII occupancy defects to *atntr1* mutants. Inhibition of PolII elongation by 6AU or inhibition of PolII endonucleolytic cleavage in *atntr1* mutant leads to partial reversal of the splicing defects.**

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**Introduction**

Splicing is a highly complicated process that involves more than 200 proteins and five small RNAs associated with the spliceosome at different stages of splicing (Wahl *et al*, 2009). This enormous number of proteins is reflected in the number of molecular processes, including transcription, that are intertwined with splicing. Alternative splicing is a manifestation of this vast complexity, with more than 95% of human (Pan *et al*, 2008) and 60% of *Arabidopsis* (Marquez *et al*, 2012; Filichkin *et al*, 2009) genes showing at least two splicing isoforms.

One of the key plant developmental regulators with reported alternative splicing of its pre-mRNA is the DELAY OF GERMINATION 1 protein, DOG1 (Bentsink *et al*, 2006). The *DOG1* expression level is responsible for the delay in germination in freshly harvested seeds and is regulated by different transcription elongation factors (Liu *et al*, 2007; Grasser *et al*, 2009), making it a good plant model for studying the crosstalk between splicing and elongation.

The interconnection of transcription and splicing has been extensively studied (Howe *et al*, 2003; Chanarat *et al*, 2011; Close *et al*, 2012; Pagani *et al*, 2003). Several models have been proposed to explain how chromatin regulates alternative splicing, including the direct sensing of histone markers by spliceosome-associated factors and the effect of the transcription elongation rate on alternative splice site selection. This latter model is known as a kinetic coupling model (de la Mata *et al*, 2003) and is based on the observation that the modulation of RNA polymerase II (PolII) elongation affects the selection of alternative splice sites: the slowing down of polymerase leads to exon inclusion and upstream splice site selection events, and the acceleration of PolII elongation leads to exon skipping and downstream splice site selection. Recent splicing analysis in an broad list of yeast PolII mutants with slow and fast elongation kinetics has confirmed the original model (Braberg *et al*, 2013). Although the slow kinetics of PolII leads mainly to exon inclusion there are several reports where reduced PolII elongation results in increased alternative exon skipping (Dutertre *et al*, 2010; Ip *et al*, 2011). Extensive molecular characterization, of slow PolII elongation dependent CFTR alternative exon 9 skipping, reviled role of PolII kinetics in control of negative splicing regulator binding (Dujardin *et al*, 2014).

Genetic analyses have suggested that *DOG1* is a direct target of TFIIS (Mortensen & Grasser, 2014). TFIIS is an elongation factor (Sekimizu *et al*, 1976) required for RNA polymerase II processivity both *in vitro* (Cheung & Cramer, 2011; Izban & Luse, 1992; Reines, 1992) and *in vivo* (Sigurdsson *et al*, 2010), and is important for the splicing of a reporter gene in yeast (Howe *et al*, 2003). Interestingly, human TFIIS is able to overcome PolII pausing at alternative exons induced by the DNA-binding protein CTCF (Shukla *et al*, 2011).

In yeast the use of a synchronised reporter system has allowed the visualisation of transient, splicing-dependent hyper-accumulation of a paused polymerase at the intron. This transient transcriptional pausing event was suggested to constitute a quality checkpoint imposed by co-transcriptional splicing (Alexander *et al*, 2010). This interpretation is consistent with the observed over-accumulation of PolII in humans at alternative introns and exons (Batsché *et al*, 2005; Saint-André *et al*, 2011) that are believed to be spliced co-transcriptionally (Pandya-Jones & Black, 2009) and are highly sensitive to the modulation of PolII kinetics, as predicted by the kinetic model.

NTR1 is an accessory spliceosomal component that has been characterised as an interactor of the NineTeen Complex (NTC) in yeast (Tsai, 2005; Agafonov *et al*, 2011). NTR1 increases PRP43 helicase activity, facilitating intron lariat release. In addition, NTR1 has been proposed to assist in the PRP43-dependent spliceosome quality checkpoint throughout the splicing cycle (Koodathingal *et al*, 2010; Mayas *et al*, 2010).

In agreement with this, NTR1 has been repeatedly co-purified with the spliceosome at different stages of splicing (Cvitkovic & Jurica, 2013). The spliceosome complex has not been purified in plants, but the *Arabidopsis* spliceosomal timekeeper locus 1 protein (STIPL1) has been characterised as a plant NTR1 homologue (Jones *et al*, 2012). In agreement with this interpretation, a mutant of the *Arabidopsis* NTR1 homologue has extensive splicing defects and show circadian clock defects due to the missplicing of one of the circadian clock genes (Jones *et al*, 2012). Surprisingly, purification of the human NTR1 complex has revealed that in addition to its interaction with PRP43, it is co-purified with members of conserved GCFC (GC-rich sequence DNA-binding factor-like protein) domain-containing proteins: C2ORF3 and GCFC (Yoshimoto *et al*, 2014). The closest *Arabidopsis* homologue of C2ORF3 and GCFC is ILP1, a protein shown to bind DNA and controls endoreduplication (Yoshizumi *et al*, 2006).

Our study shows that the *Arabidopsis thaliana* NTR1 homologue (AtNTR1) is crucial for *DOG1* expression and splicing. Analysis of splicing defects at *DOG1* and other genes show a strong bias towards downstream splice site selection in *atntr1*. In accordance with kinetic coupling model we hypothesise that this bias is a consequence of fast PolII elongation at the splice sites in *atntr1* mutant. Our PolII ChIP data showed a localised decrease in PolII occupancy at affected splice sites, this result is interpreted by us as a localised change in elongation rate*.* However, it was not the case for two other splicing factors or in plants with chemically inhibited splicing. This interpretation is consistent with observed immuno co-localisation of AtNTR1 with PolII in the nucleus, and the presence of AtNTR1 at DNA of its target genes as shown by ChIP. Analysis of AtNTR1 dependent splicing events showed that NTR1 is required for splicing of strong, consensus like, alternative splice sites. This was corroborated by mutational analysis that showed an *atntr1* dependent increased accumulation of PolII ChIP signal at the strong alternative splice sites.

Our data are consistent with NTR1 being required for co-transcriptional pausing of polymerase at strong alternative splice sites. We interpret the directionality of alternative splicing defects in *atntr1* mutant as a manifestation of PolII elongation defects. The role of transcription elongation in alternative splice site selection has been extensively studied (de la Mata *et al*, 2011). To asses if alternative splice site selection in plants is also dependent on transcription elongation rate we have so far indirectly compromised PolII endonucleolytic cleavage by mutating TFIIS and by exposing the plants to 6AU and MPA treatment. Alternative splicing changes observed were mostly consistent with prediction form the kinetic coupling model and opposite to one observed in *atntr1* mutant (de la Mata *et al*, 2003) supporting our conclusions.

**Results**

**AtNTR1 regulates seed dormancy, *DOG1* expression, and splicing**

The NTR1 homologue in *Arabidopsis* was originally identified as a result of a genetic screen for circadian clock regulation (Jones *et al*, 2012). We found that in addition to its role in circadian clock, *atntr1-1* mutants showed pleiotropic phenotypes, including low seed dormancy, altered flowering time, altered leaf morphology, and enhanced lethality at elevated temperatures (Figure 1A, Supplementary Figure S1). We focused on the seed dormancy phenotype and confirmed that both available alleles, *atntr1-1* and *atntr1-2*, showed enhanced germination without stratification (Figure 1A and 1B, Supplementary Figure S1A). Interestingly, *dog1-1* mutants have been shown to have similar phenotype (Bentsink *et al*, 2006). Consistent with the low seed dormancy phenotype of *atntr1* mutants, we found that *DOG1* gene expression was reduced in seeds (Figure 1C) and seedlings (Supplementary Figure S1B). Because AtNTR1 deficiency leads to massive splicing defects in *Arabidopsis* (Jones *et al*, 2012) and *DOG1* is a subject of alternative splicing (Bentsink *et al*, 2006), we analysed the splicing defects of the *DOG1* transcripts. The *atntr1-1* mutation resulted in more pronounced usage of the 5’ downstream splice site, with a concomitant reduction in the upstream 5’ SS selection in comparison with wild type plants (Figure 1D). Additionally, an approximately 50% increase in intron retention was observed (Figure 1D). The altered splicing isoforms corresponded to the most abundant of the four reported splice isoforms of *DOG1*, namely alpha and beta. We therefore measured all four isoforms reported for *DOG1* and found that, indeed, isoforms alpha and beta were the most affected (Supplementary Figure S1C, D) (Bentsink *et al*, 2006; Schwab, 2008).

**The *atntr1* mutant shows bias in alternative 5’ and 3’ splice site selection**

We were intrigued by the change in splice site selection on *DOG1* towards the downstream splice site, as *DOG1* expression strongly depends on factors required for efficient transcriptional elongation including TFIIS, and tendency toward downstream splice site selection could indicate a defect in the PolII elongation rate (Mortensen & Grasser, 2014; Liu *et al*, 2007). We therefore extended our observation of alternative splicing defects. Independently of the previous report, a selection of 144 alternative splice events in *atntr1-1* were analysed (Jones *et al*, 2012). In agreement with previous results, we found that the most abundant splicing defects were intron retention and exon skipping (Figure 1E) (Jones *et al*, 2012). For 144 alternative splicing events analysed, 74 were significantly changed. In agreement with the directionality of splice site selection on *DOG1* a prominent bias in the directionality of alternative 5’ splice site selection was observed (Figure 1D, E). Of 16 affected alternative 3’ splice site selection events, 14 (88%) were changed in *atntr1-1* towards downstream splice sites (SS). Similar bias was also observed in the directionality of splicing events in the case of 5’ splice site events (7 of 10 changed towards downstream SS -70%) and in exon skipping events (18 of 20 changed towards exon skipping - 90%) (Figure 1E and Supplementary Table S1).

Upstream/downstream splice site selection has been proposed to represent a manifestation of the polymerase II elongation rate (de la Mata *et al*, 2003, 2011). Therefore, the bias we observed could indicate a defect in transcription elongation across the affected splice sites in the *atntr1-1* mutant.

**AtNTR1 is required for splicing of strong consensus splice sites**

Next we wanted to understand what creates specificity for NTR1 at some splice sites but not the others. Analysis of acceptor splice sites consensus sequence showed no clear difference between AtNTR1dependent and independent introns (Supplementary Figure S2B). On the other hand, analysis of donor site revealed a significant difference (*p*-value > 0,05 Fisher's exact test) at position +3/+4. AtNTR1 dependent introns show a higher likelihood of A/G at +3 and A at +4 positions compared to AtNTR1 independent splice sites (Supplementary Figure S2A). Given the consensus donor splicing site sequence in *Arabidopsis* is AG|GTAAGT, affected introns more closely resemble the whole genome consensus than introns with splicing unaffected in *atntr1-1*. The AA nucleotides at position +3/+4 of the 5` SS consensus are involved in base paring with U1. During the splicing cycle the base paring with U1 has to be exchanged for U6. It is interesting to note that U6 in *Arabidopsis* does not form base paring with canonical positions +3/+4 of the consensus sequence.

We decided to test if the NTR1 requirement for splicing is specified by the strong sequence of alternative splicing donor splice site as has been suggested by our consensus analysis. Therefore, an alternative 5`SS event that is not dependent on NTR1 and display week consensus sequences at the upstream and downstream sites was selected. Then, we mutated those sites into strong consensus like sequences by changing two nucleotides at each site (Supplementary Figure S1C). Analysis showed that whereas splicing of the native version (5`SS wt) was not changed in *atntr1* mutant the splicing of the mutated construct (5'SS strong) was affected (Supplementary Figure S1D). Although the change in alternative splice site selection was small, it was statistically significant (p-value 0.01). This confirms our initial observation that the 5`SS consensus with extended homology to U1 snRNA constitutes a preferable target for NTR1 splicing activity. In addition, this analysis shows that the AtNTR1 effect on splicing is downstream of splice site recognition by the spliceosome, which is consistent with the AtNTR1 role in recycling of U6.

**In addition to U6/5/2 snRNPs, AtNTR1 interacts with U1**

To obtain more insight into the potential function of AtNTR1, RNA molecules associated with AtNTR1 were analysed using RNA immuno-precipitation (RIP) followed by RT-PCR. In this experiment we used a complementing transgenic *Arabidopsis* line expressing the AtNTR1-GFP fusion protein in the *atntr1-1* genetic background and antibodies recognizing GFP. Given the well documented role of NTR1 in U6, U5 and U4 recycling we tested AtNTR1 interaction with those molecules. We could clearly observe an enrichment of U6, U5, and U2 RNA in the AtNTR1-GFP-immunoprecipitated fraction, compared to our negative control (Figure 2A). Surprisingly, a strong and reproducible interaction of AtNTR1 with U1 was also observed (Figure 2A). In contrast, U3 and 18S rRNA showed no enrichment, confirming the stringency of our method (Figure 2A). This result was further confirmed by our mass spectrometry-based purification of AtNTR1-associated proteins, as the U1 associated U1A protein was one of our highest-scoring interactors (Table 1).

**ILP1, a GCFC domain-containing protein, interacts with AtNTR1 and is required for efficient splicing**

The highest-ranking NTR1 interactor on our list was a protein known as ILP1 in *Arabidopsis* (Yoshizumi *et al*, 2006). ILP1 contains the GCFC domain (GC-rich sequence DNA-binding factor-like domain) and is a homologue of the human proteins C2ORF3 and GCFC (also known as Pax3/7BP) (Yoshimoto *et al*, 2014; Diao *et al*, 2012). Both, the bimolecular fluorescence complementation assay (BiFC) using YFP (Supplementary Figure S3B) and an yeast two-hybrid assay (Supplementary Figure S3C) confirmed our original finding and suggested direct AtNTR1-ILP1 interaction. Moreover, homologues of ILP1 in humans co-purify with TFIP11, a human homologue of AtNTR1 (Yoshimoto *et al*, 2014), indicating that this interaction is conserved between species, which suggests that it may be important for NTR1 function. ILP1 in *Arabidopsis* binds to a promoter of a key cell cycle gene and controls its expression, providing a possible explanation for endoreduplication defects in the *ilp1* mutant (Yoshizumi *et al*, 2006). In addition, human homologs of ILP1 likewise bind to gene promoters to regulate their expression (Diao *et al*, 2012).

Next, we tested whether ILP1 was involved in splicing regulation in *Arabidopsis*. The *atntr1-1* and *ilp1-1* mutants were analysed side by side, and *ilp1-1* had very strong splicing defects, with virtually all splicing events affected in *atntr1-1* also being misregulated in the *ilp1* mutant (Supplementary Figure S3D, Supplementary Table S2). This finding is consistent with the recent data on a human ILP1 homologue showing that the depletion of C2ORF3 by RNAi repressed pre-mRNA splicing in vitro (Yoshimoto *et al*, 2014).

We conclude that ILP1, like its human homologues, is a direct interactor of AtNTR1 and that GCFC domain-containing proteins are required for efficient splicing both in *Arabidopsis* and in humans.

**PolII co-localises with AtNTR1**

AtNTR1 immunolocalisation was investigated, to test the relationship between AtNTR1 and PolII. We confirmed previous results from human cells showing that NTR1 is localised in the nucleus but excluded from the nucleolus, using a complementing genomic NTR1-GFP line (Supplementary Figure S1I,J) and AtNTR1 antibody (Figure 2B) (Tannukit *et al*, 2008). In addition, using dual labelling, it was found that like its mouse homologue, AtNTR1 is only partially co-localised with the SC35 splicing factor (Figure 2B) (Wen *et al*, 2005). Subsequently, we investigated the co-localisation of AtNTR1 with PolII. Three different PolII antibodies were used: the first recognising all forms of PolII (total PolII), the second recognising the Ser5-phosphorylated form of PolII (PolIIA) that is usually associated with the initiation of transcription, and the third recognising Ser2-phosphorylated PolII (PolIIO), which is believed to primarily mark actively transcribing PolII associated with gene bodies. Partial co-localisation with total PolII was observed that could be attributed to the Ser2-phosphorylated form of PolII, as we observed no co-localisation with PolIIA and strong co-localisation of AtNTR1 with the PolIIO (Figure 2B). Although the functional distinction between Ser5-phosphorylated and Ser2-phosphorylated PolII is not absolute it is generally believed that Ser2-phosphorylation is a mark of elongating polymerase.

**AtNTR1 acts co-transcriptionaly at affected splice sites.**

The co-localization of AtNTR1 with Ser2 phosphorylated PolII suggested that NTR1 can be physically present at target genes. In order to test this, we analyzed AtNTR1 localization on the *DOG1* gene by chromatin immunoprecipitation (ChIP), using antibodies that recognise AtNTR1.

Our ChIP analysis shows that in contrast to an intergenic region selected as a negative control, AtNTR1 is present at the gene body and promoter of *DOG1* (Figure 3). In addition, a set of 5 other genes were tested for NTR1 presence. Clear NTR1 signal could be detected on all of them (Supplementary Figure S4). Target genes were selected from *atntr1* misregulated alternative splicing sites containing genes to represent different types of alternative splicing events. The physical presence of AtNTR1 on those genes substantiates our NTR1 PolII co-localisation data and suggests that at least some of NTR1 activity is happening co-transcriptionaly.

We conclude for all tested genes where AtNTR1 is required for splicing it is present at, or close to, DNA. In addition, our ChIP analysis suggests that AtNTR1 is present throughout the analysed genes, with no clear enrichment at misspliced introns. This result is consistent with our immuno co-localisation of AtNTR1 and PolII.

**Localized PolII level reduction in *atntr1* mutant**

Our data show a strong link between AtNTR1 and PolII. In addition, the splicing defects observed in the *atntr1-1* mutant are interpreted by us as a manifestation of fast PolII elongation across the splice sites. We therefore considered a possibility that AtNTR1 is involved in the control of PolII elongation at splice sites. To start to address this, the genes which we previously showed to be direct targets of AtNTR1, where used to assess PolII profile by ChIP using antibodies that recognise total PolII.

Analysis of PolII occupancy in *atntr1-1* consistently revealed a significant reduction of PolII for all genes with AtNTR1 dependent splicing (5 of the 5 genes tested) (Figure 4 and Supplementary Figure S5). With an exception of *DOG1*, the reduction was limited to or strongest at the regions of misspliced introns. The *At5g04430* gene was analysed as control as it shows *atntr1* independent alternative splicing and consistently showed no significant reduction in PolII levels in *atntr1* mutant.

The localised decrease in PolII occupancy in *atntr1* mutant we observe could be interpreted in many ways. Given the directionality in splice site selection we favour an interpretation that it represent a manifestation of a localised change in elongation rate in *atntr1* mutant across the affected splice sites.

**Reduction in PolII levels in *atntr1* is not a consequence of reduced splicing efficiency**

Published examples from yeast suggest that aberrant splicing in tested splicing mutants leads to stronger PolII pausing (Alexander *et al*, 2010; Chathoth *et al*, 2014). In the case of *atntr1* we observe the reduction in PolII accumulation across the intron associated with aberrant splicing. It is, possible however that the reduction of PolII levels observed in *atntr1* could be a consequence of general splicing malfunction in this mutant. To validate this possibility we tested if PolII occupancy defects observed in *atntr1* could be mimicked by inhibition of splicing. We first used a chemical inhibitor of splicing, Herboxidiene. Herboxidiene is a plant herbicide, , that was shown to inhibit splicing in humans by direct binding of the human SAP155 splicing protein (Hasegawa *et al*, 2011; Miller-Wideman *et al*, 1992; Lagisetti *et al*, 2014).

Our results clearly show a marked inhibition of splicing across nearly all analysed splicing events (Figure 4, Supplementary Figure S5 and Supplementary Table 3) for WT plants treated with Herboxidiene. However, the PolII ChIP occupancy analysis in Herboxidiene treated plants failed to show any significant reduction in PolII levels at the affected splice sites (Figure 4, Supplementary Figure S5). Herboxidiene is inhibiting splicing in human cells by binding the same splicing factor as Spliceostatin A (Kaida *et al*, 2007). It is therefore interesting to note that our data are in agreement with data from studies in human where Spliceostatin A treatment did not cause a change in PolII levels or elongation kinetics at affected splice sites (Brody *et al*, 2011). Taken together, our results suggest that the reduction of PolII levels in *atntr1* is not a consequence of misregulated splicing but rather a direct affect of NTR1 deficiency.

In addition to chemical inhibition of splicing, we tested 2 splicing mutants known to regulate alternative splicing: *sr45-1* and *smd3-b* (Palusa *et al*, 2007; Swaraz *et al*, 2011). Our analysis showed no change in PolII occupancy on any of the 6 genes that were tested (Supplementary Figure S6). In contrast, splicing analysis revealed a substantial change in splicing of those genes that was often similar to change observed in the *atntr1* mutant (Supplementary Figure S6 and Supplementary Table 4). This shows that the PolII occupancy change observed in *atntr1* is not shared among all splicing factor mutants but is specific for the mutant lacking NTR1.

The reduction in PolII level in *atntr1* is in most cases localised to the vicinity of affected splice sites and does not extend on the whole gene, therefore is unlikely to represent a reduction in overall transcription rate. The PolII occupancy analysis in the *atntr1* mutant supports our model ofNTR1 as a component of the splice site dependent pausing mechanism at alternative splice sites in *Arabidopsis*. Alternative splice sites have been shown to accumulate high PolII signal in humans (Saint-André *et al*, 2011). We however could not detect a significant increase in the PolII signal at alternative splice sites when compared to surrounding regions in WT plants suggesting that there are many parallel mechanisms controlling PolII occupancy at those genes.

**AtNTR1 induces pausing of PolII at strong alternative splices sites on the transgene**

We previously showed that splicing of our minigene became NTR1 dependent in the construct mutated to resemble the NTR1 splice site consensus sequence. Next, we wanted to test how this is related to PolII occupancy. To do this we first analysed transgenic lines with WT alternative 5` donor splice sites (5`SS wt) and mutated NTR1 dependent strong upstream - strong downstream splice sites (5`SS strong). The 5`SS strong transgene compared to 5`SS wt transgene showed a significantly higher PolII levels, specifically at the splice site studied (Figure 5). The 5`SS strong construct differs from the 5`SS wt construct only by 2 nucleotides mutated at upstream and downstream 5`SS, respectively. The dependence, of the localised increase, in PolII occupancy, on presence of strong alternative splice sites in our minigene suggests an PolII pause site controlled by the spliceosome operates at strong alternative splice sites on our transgene.

We therefore tested if this PolII pausing on our minigene is NTR1 dependent. Analysis in the *atntr1-1* background showed a marked reduction in PolII levels for 5`SS strong but not for the 5`SS wt construct (Figure 5). Those results confirmed that AtNTR1 is required for high PolII occupancy observed on the transgene. We interpret this localised PolII occupancy change as an AtNTR1 dependent transcriptional pausing at strong alternative splice sites on our transgene.

At endogenous targets we did not observe a clear increased PolII occupancy at alternative splice sites in WT plants when compeered to surrounding regions. Therefore we can only speculate that on endogenous genes, similarly to our transgene strong alternative splice sites constitute a transcriptional pause site, but other PolII elongation control mechanism mask the result. This interpretation is consistent with the observed reduction of PolII occupancy level observed in *atntr1* mutant at endogenous targets.

The locally increased PolII occupancy observed at 5`SS strong compared to 5`SS wt construct together with localised reduction of PolII in *atntr1* both at transgene and endogenous targets suggest a role of AtNTR1 in transient transcriptional pausing at splice sites. Using chemical or genetic interference we could not induce the PolII decrease observed in *atntr1* mutant, Therefore, we interpret the PolII changes detected in *atntr1* not as a consequence of aberrant splicing but rather as a manifestation of AtNTR1 function.

There are several predictions that can be made based on our results. First, in the interpretation of splice site selection directionality in *atntr1* we assumed the existence of kinetic coupling of splicing and transcription in plants as was proposed for other organisms (de la Mata *et al*, 2011). Second, we interpret some of *atntr1* splicing defects as a manifestation of PolII elongation defects, therefore at least some of the defects should be reversed by slowing down PolII elongation.

**The dominant-negative TFIIS mutant blocking PolII endonucleolytic cleavage show upstream splice site selection**

The directionality of alternative splicing site selection in *atntr1* is interpreted by us as consequence of PolII elongation defect in *atntr1* suggesting existence of kinetic coupling between transcription and splicing as shown before in yeast and humans (de la Mata *et al*, 2011). The kinetic coupling of transcription and splicing in yeast and humans has been initially proposed based on directionality in splice site selection in fast and slow elongating PolII mutants. Given that *DOG1*, one of our target genes, has been shown to be regulated directly by TFIIS we focussed our attention on this mutant (Mortensen & Grasser, 2014). TFIIS is a conserved component of the PolII holoenzyme required for efficient PolII endonucleolytic cleavage activity (Sigurdsson *et al*, 2010). Therefore, TFIIS has a modulatory function in enhancing PolII processivity, allowing reinitiation of backtracked, paused polymerase. TFIIS mutants have been shown to be involved in splicing in yeast and humans (Howe *et al*, 2003; Shukla *et al*, 2011).

We expected that *tfIIs* knockout plants will show enhanced pausing of PolII, which in turn would be reflected in alternative splice site selection. Our alternative splicing analysis in *tfIIs* knockout showed only mild defects in splicing, with 10 of 284 splicing events (including the set analysed for AtNTR1) being affected (Supplementary Figure S7D, Supplementary Table S5). We therefore constructed an enhanced TFIIS mutant in *Arabidopsis* (TFIISmut) by mutating two key amino acids in the TFIIS trigger loop, as was described for yeast (Sigurdsson *et al*, 2010) (Supplementary Figure S7A). These mutations resulted in a protein that not only is unable to activate PolII endonucleolytic cleavage but also blocks it, as was shown in vitro (Sigurdsson *et al*, 2010). We were unable to recover any transformants when TFIISmut construct was expressed the in the *tfIIs* mutant, suggesting that PolII endonucleolytic cleavage is required for the viability of *Arabidopsis*, which is in agreement with findings concerning *Saccharomyces serevisiae*. As in the case of the yeast, however, it was possible to obtain viable transformants when we combined the TFIISmut-expressing construct with the WT copy of TFIIS. These transgenic plants showed a range of developmental defects, including leaf serration and reduced steam elongation (Supplementary Figure S7B). We conclude that, similar to *S. serevisiae*, the TFIISmut protein shows a dominant-negative phenotype.

Next, splicing analysis was repeated in TFIISmut using the same set of splicing events that were previously analysed for the *tfIIs* knockout allele. We found that 62 out of 284 alternative splicing events showed misregulation of splicing (Figure 6, Supplementary Figure S7, Supplementary Table S5). For the majority of these cases, including *DOG1*, we observed a preferential selection of the upstream 5’ splice site (9 out of 11 cases) (Figure 6A, B). In addition, a strong tendency toward enhanced intron splicing in the case of intron retention events (11 out of 14) and enhanced exon inclusion in the case of exon skipping (14 of 18 events) was observed (Figure 6B). This type of bias toward upstream splice sites selection, enhanced intron splicing, and exon retention has been postulated to result from the kinetic coupling of splicing and transcriptional elongation (de la Mata *et al*, 2003, 2011). In summary, of 62 affected alterative splicing events in TFIISmut, 45 changed in the direction predicted by the kinetic model. Our data therefore, although did not explicate confirmed the existence of kinetic coupling of splicing and transcription elongation in plants, is consistent with it operating in plants.

Most importantly, the bias in the direction of splice site selection in the TFIISmut background was predominantly opposite to the changes observed in *atntr1-1* mutant plants (compare Figure 6B and Figure 1E). 72.5% of the splicing events changed in both TFIISmut and *atntr1* were affected in opposite directions (Figure 6C). These data therefore corroborate our interpretation of directionality in *atntr1* alternative splice site selection as a manifestation of PolII elongation defects.

**PolII profiling reveals an enhanced PolII pausing at affected introns in TFIISmut**

The opposite splice site selection preference observed in TFIISmut and *atntr1* is consistent with our model of NTR1 dependent transcriptional pausing at the splice sites. It also suggested that TFIIS mutation lead to enhanced PolII pausing at the splice sites. As a consequence this pausing would be manifested in preference for upstream splice site selection in TFIISmut. To test this hypothesis a set of 6 genes was chosen for which, we previously shown that they are direct targets of AtNTR1, and are oppositely spliced in *atntr1-1* and TFIISmut (5 out of 6). This set was profiled for PolII occupancy in TFIISmut and *atntr1-1* mutants grown side by side. We found that TFIISmut consistently showed higher total PolII occupancy in our assay at the alternatively spliced junction (5 of 6 genes tested) (Supplementary Figure S8). This result showed that the splicing defects observed in TFIISmut are at least correlated with increased pausing of PolII in TFIISmut in the context of affected introns.

Previous reports indicated that aberrant splicing leads to accumulation of PolII at introns. At least in yeast, this paused PolII is phosphorylated at Ser5 on the CTD tail by an uncharacterised kinase (Alexander *et al*, 2010). We therefore analysed the phosphorylation status of the PolII CTD tail at Ser5 in *atntr1-1* and TFIISmut plants at our gene targets. We found that, indeed, the polymerase showed a predicted change in Ser5 phosphorylation that followed the changes observed in the total PolII level, suggesting that the majority of polymerase paused at those introns was Ser5-phosphorylated (Supplementary Figure S8).

**Inhibiting transcription elongation by compromising PolII endonucleolytic cleavage or 6AU treatment partially reverses some of *atntr1* splicing defects**

Next, we wanted to substantiate our model that *atntr1* splicing changes are a consequence of PolII elongation defects caused bythe lack of AtNTR1. We assumed that if this is true then slowing down of PolII should reverse some of *atntr1* splicing defects. Analysis of splicing in *atntr1-1*TFIISmut double mutant reviled a clear but only partial reversal of *atntr1* splicing for some of the gene studied. 2 out of 5 genes studied including *DOG1* showed a shift in splice site selection towards the WT in the double mutant (Supplementary Figure S9, Supplementary Table S6). As only partial reversal at 2 out of 5 genes tested (or 3 out of 10 splicing isoforms changed in single mutants with p-value < 0.05 and delta ≥5%) could be observed, we extended our set to 45 splicing isoforms (in 14 different genes). Of those 45 analysed 18 did not show any reversal in the *atntr1-1*TFIISmut double mutant compared to *atntr1-1*, and 27 did show partial reversal of splicing (Supplementary Figure S9). 10 of those 27 changed events showed significant change with p-value < 0.05 and delta ≥5% when compared directly between the *atntr1-1*TFIISmut and *atntr1-1* single mutant. TFIISmut expression analysis confirmed that the double mutant expressed our transgene at similar level to WT plants (Supplementary Figure S9)

To further substantiate those observations we have used 6AU to challenge transcription elongation in plants, and analyzed the alternative splicing of a selected set of genes. We have found that 6AU and MPA clearly affects splice site selection in plants. For some of the genes analysed the pattern is opposite to pattern in *atntr1* mutant (3 out of 6) and agree with the prediction of the kinetic coupling model (Supplementary Figure S10). What’s more, analysis in *atntr1* mutant treated with 6AU showed that even for events where 6AU effect in WT plants cannot be seen so clearly the 6AU partially or fully reverses the *atntr1* splicing defects (compare column 1 and 2 on panel B Supplementary Figure S10). Out of 12 events significantly changed in comparison of *atntr1* and *atntr1* treated with 6AU, 10 events are partially or fully reversed in the *atntr1* 6AU treated plants, one event changes in the same direction as in *atntr1* compared to WT and 1 event was not changed in *atntr1* compared to WT so cannot be classified (Supplementary Figure S10B, third column, events labelled with stars indicate t-test p-value less than 0.01).

We therefore concluded that indeed some of the splicing defects in *atntr1* are likely to be a consequence of elongation defects and as such can be reversed by interference with PolII elongation by 6AU or inhibition of PolII endonucleolytic cleavage.

***atntr1* shows low sensitivity to 6AU mediated grow inhibition**

Our data provide several lines of evidence for the role of *atntr1* in transcription elongation control at splice sites. In yeast, viability test in the presence of 6AU, has been extensively used to characterise transcription elongation deficient mutants (Riles *et al*, 2004). 6AU treatment leads to reduction in *in vivo* nucleotides levels that causes transcriptional elongation to be more dependent on a fully functional RNA polymerase (Exinger & Lacroute, 1992). We therefore have subjected WT, *tfIIs* and *atntr1* mutants to the 6AU treatment. Phenotype analyses showed that *tfIIs* mutant is indeed highly sensitive to 6AU as expected from numerous reports in yeast. In contrast, *atntr1* is strongly resistant to growth inhibition by 6AU (Supplementary Figure S11). The molecular mechanism of 6AU sensitivity in plants has not been studied but the opposite sensitivity of *atntr1* and *tfIIs* mutants to 6AU is reminiscent of opposite directionality in alternative splice site selection and PolII occupancy defects. We therefore interpret the lower sensitivity of *atntr1* to 6AU as an indication of AtNTR1 negative role in transcription elongation control in plants.

**Discussion**

NTR1 was initially characterised as a spliceosomal disassembly factor, and substantial evidence from both humans and yeast supports its crucial function in this process (Yoshimoto *et al*, 2014; Tsai, 2005). In *Arabidopsis*, STILP1 has been described as a NTR1 homologue required for the correct splicing of a range of targets (Jones *et al*, 2012). Here, we report that AtNTR1 (STILP1) mutants in *Arabidopsis*, in addition to circadian clock defects, show a pleiotropic phenotype, including weaker seed dormancy and a concomitant reduction in the expression of a key seed dormancy regulator, *DOG1*.

**NTR1 function in splicing**

In agreement with the predicted function of NTR1 in splicing, we found that *atntr1* mutants show splicing defects in a wide range of genes, including *DOG1*, where we observe changes in alternative splicing. Although the formal role of NTR1 in spliceosomal disassembly in *Arabidopsis* *thaliana* remains unproven, we provide evidence for the interaction of *Arabidopsis* NTR1 with U6 snRNP, which is consistent with its function in U6 recycling in yeast and humans (Boon *et al*, 2006; Yoshimoto *et al*, 2014).

In agreement with data for human homolog of NTR1 we show evidence for *in vivo,* direct interaction between AtNTR1 and GCFC domain containing protein ILP1. It is interesting to note that both the *Arabidopsis* and human NTR1-interacting GCFC proteins are associated directly with gene promoters and regulate gene expression (Yoshizumi *et al*, 2006; Diao *et al*, 2012). Therefore, one possibility is that GCFC domain containing proteins provide target specificity for NTR1 proteins. However, the strong interdependence of the NTR1 and GCFC proteins in efficient splicing, shown by us here, is in disagreement with this model and suggests a role of GCFC proteins as spliceosomal factors.

Our consensus analysis of NTR1 dependent splice sites showed that NTR1 is predominantly required for efficient splicing of strong, consensus like, alternative splice sites. The dependence of strong alternative splice sites on NTR1 may be explained by the U1/U6 exchange defect (Konforti *et al*, 1993). It is interesting to note that the difference between NTR1 dependent and independent splice sites lays in nucleotides forming extended interactions with U1 but not U6. Therefore the NTR1 dependent splice sites are more efficiently bound by the U1 and may require a relatively high U6 levels or remodeling activity to exchange the U1 for the U6 RNA. Defects in NTR1 in yeast has been shown to lead to reduction in free U6 levels providing an explanation for the NTR1 specificity we observe (Boon *et al*, 2006).

**NTR1 function in PolII transcription**

In addition to the involvement of NTR1 in splicing, we provide several lines of evidence for additional transcription-related functions of NTR1.

Careful analysis of splicing defects, including splicing defects on *DOG1*,in *atntr1* reveals a bias in alternative splice site selection that is indicative of fast PolII elongation across splice sites. AtNTR1 ChIP data showed that NTR1 is physically located at the target splice sites and that it deficiency leads not only to splicing defects but also to locally reduced PolII levels at those splice sites. We interpret this localised PolII occupancy change as faster transcription elongation across those sites in *atntr1*. This interpretation is corroborated by several lines of evidence. First, the localised PolII occupancy decrease would be difficult to reconcile with a transcriptional change, where PolII levels should be altered globally and not locally. Second, the opposite change in PolII occupancy and directionality of alternative splice site selection between TFIISmut and *atntr1* suggests AtNTR1 has opposite function to TFIISmut in transcription elongation control. Consistent with this interpretation our transgen analysis clearly showed that on this target strong alternative splice sites do show high PolII accumulation that is dependent on AtNTR1. In line with the role of NTR1 in transcription elongation control in plants we show a reduced sensitivity of *atntr1* mutant to 6AU mediated grow inhibition.

The directionality in alternative splice site selection we observe in *atntr1*, TFIISmut and 6AU/MPA treated plants are mostly consistent kinetic coupling of transcription and splicing (de la Mata *et al*, 2003). The kinetic coupling has to our knowledge never been shown to operate in plants. Our data do not provide a direct confirmation of the kinetic coupling operating in plants but are consistent with this interpretation.

Splicing analysis in *atntr1*TFIISmut double mutant showed that some of the splicing defects including defects observed at the *DOG1* gene can be partially reversed. Similar reversal of *atntr1* splicing defects was observed after *atntr1* 6AU treatment suggesting that this reversal can be attributed to slow down of transcription elongation. This can be interpreted as a compensation of *bona fide* splicosomal defects by extension of the time window for splicing. The other possibility, that we favour, is that some of the splicing defects in *atntr1* are due to faster transcription elongation. Therefore slowing down the elongation rate by 6AU or interference with PolII endonucleolytic cleavage reverses those splicing defects. Our splicing assay allows for the simultaneous detection of several missplicing events of one gene. Interestingly, in the case of *DOG1* and At4g36690 the 3`splice site selection event showed partial reversal in *atntr1*TFIISmut double mutant background. However the intron retention event was fully insensitive to TFIISmut, even thou it was responding to TFIISmut in WT backgrounds (Supplementary Figure S9A). The concurrence at the same intron of splicing events that can and cannot be reversed by TFIISmut in the *atntr1* background, even though they showed same sensitivity to TFIISmut in the WT background, suggest to us that the reversible splicing events are a consequence of elongation defects in *atntr1* rather than the spliceosome dysfunction.

In yeast, introns are sites of transient PolII pausing induced by splicing itself rather than recruitment of the spliceosome (Alexander *et al*, 2010). This pausing has been proposed to constitute a quality checkpoint for splicing, which provides feedback on transcription. Recently the role of CUS2 protein as a transcriptional roadblock has been established (Chathoth *et al*, 2014). We have not addressed the role of CUS2 in transcriptional pausing in *Arabidopsis* but our data show an analogous role of AtNTR1 in this process. The role of NTR1 in transcription elongation control in yeast has also not been addressed but it is interesting to note that NTR1 in yeast, in contrast to all other higher eukaryotes, lacks several protein domains. One interpretation is that in higher eukaryotes the increased splicing complexity including occurrence of alternative splicing has lead to development of additional splicing associated transcriptional check points, including the AtNTR1 dependent.

Our chemical and genetic inhibition of splicing failed to reproduce the change in PolII levels observed in the case of *atntr1* across the splice sites. Herboxidiene and both tested splicing mutants inhibit splicing at different stages than NTR1 has been shown to act in yeast and humans. This together suggests that the localised PolII reduction observed in *atntr1* is not a consequence of inefficient splicing but rather represent a consequence of NTR1 deficiency. On the other hand, the minigene analysis showed that the NTR1 dependent PolII pausing requires a strong alternative splice site. This indicates that the strong alternative splice sites are a prerequisite for NTR1 activity both in terms of controlling splicing and in terms of controlling PolII levels at the splice sites. Our data suggest that NTR1 helps to create a pause site for elongating RNA polymerase II at alternative introns to further assist their splicing. This interpretation is consistent with the proposed role of NTR1 in quality proofreading of splicing (Pandit *et al*, 2006; Koodathingal *et al*, 2010).

**Materials and methods**

**Plant materials, constructs, *Arabidopsis* transgenic lines and Seed dormancy test.** Wild-type Col-0 plants, *atntr1-1* (SALK\_073187), *atntr1-2* (GABI\_852B07), *tfIIs-2* (SALK\_027259), *ilp1-1* (SALK\_030650), *ilp1-2* (SALK\_135563), *dog1-1* (SALK\_000867) mutant alleles where used. PCR based site-directed mutagenesis (Ho *et al*, 1989) was carried out to generate TFIISmut construct as shown in Supplementary Figure S4 , under a 2390bp native promoter in pCambia1300. Plants were transformation as described (Logemann *et al*, 2006). NTR-GFP lines were created by transformation (Logemann *et al*, 2006)of *atntr1-1* with ATNTR1 genomic fragment including 2070bp promoter cloned into pCambia1300, fused in frame with C terminal GFP-tag. Plants were grown (Sanyo for plates or Conviron walk in chamber for soil) under controlled environmental parameters: 70% humidity, temperature 22°C, 16 h light/8 h dark photoperiod regime at 150–200 mE/m2. For seed dormancy test, fresh harvested seeds were sterilized with 1% NaClO for 10mins and washed with distilled water for 3 times, seeds were then sown evenly on MS agar plate. Germination rate was scored daily until all genotypes reached to 100% germination. **RNA extraction and RT-qPCR.** 14-day-old seedling grown on MS plate and 16 days old siliques were harvested, frozen in liquid nitrogen immediately and stored in -80℃. RNA was extracted by hot phenol method (Shirzadegan *et al*, 1991). RevertAid Kit (Thermo scientific) was used for RT with 5μg of total RNA, after DNase treatment TURBO DNA-free (Life technologies) and oligodT primer. 1μl of RT reaction mix was used for qPCR reaction with SYBR Green I Master (Roche) using LightCycler® 480 Instrument. **Immunoprecipitation and mass spectrometry.** AtNTR1-GFP lines were used for immunoprecipitation with GFP-Trap agarose beads (Chromotek), following user manual. Eluted proteins were precipitated by acetone, and digested by trypsin (Thermo scientific). Mass spectrometry was performed in Institute of Biochemistry and Biophysics proteomics facility. Peptide mixtures were applied to RP-18 pre-column on UPLC system (NanoAcquity, Waters) using water containing 0.1% FA as a mobile phase followed by a nano-HPLC RP-18 column (75 μM, Waters) using ACN gradient (0 – 35% ACN in 160 min) in the presence of 0.1% FA at a flow rate of 250 nl/min. The column outlet was coupled directly to the ion source of Orbitrap Velos mass spectrometer (Thermo). The acquired MS/MS data were pre-processed with Mascot Distiller software (v. 2.4.3, MatrixScience) and a search was performed with the Mascot Search Engine (MatrixScience, Mascot Server 2.4) against the TAIR10 database (35386 sequences; 14482855 residues). Mascot search settings were as follows: enzyme-Trypsin, missed cleaveages-0, fixed modifications: Carbamidomethyl (C), variable modifications: Oxidation (M). **Immunocolocalization.** DNDYEGGRWEGDEFVYC and DMIDEDVEVRGGLGIGC peptides were synthesized to generate rabbit anti-AtNTR1 polyclonal antibody (Eurogentec). Immunocolocalization was performed as described (Zhang *et al*, 2013). Nuclei were fixed with 4% paraformaldehyde and blocked 15 min with 0.05% acBSA (Aurion, The Netherlands) in PBS buffer. Then nuclei were incubated in double labelling reactions with primary antibodies anti-AtNTR1 diluted 1:200 and mouse IgG [4H8] antibody (Covance) diluted 1:100 or mouse IgG anti-SC35 (Sigma) diluted 1:100, or mouse IgM anti-RNA polymerase II H14 (recognizes the phosphoserine 5 version of RNA PolII, Pol IIA) or mouse IgM anti RNA polymerase II H5 (recognizes the phosphoserine 2 version of RNA PolII, Pol IIO) (Covance) diluted 1:100 in PBS bufer with 0.05% ac BSA overnight at 8℃. Nuclei were then washed in PBS and incubated in secondary antibodies: Alexa Fluor 594 goat anti rabbit (1:500, Invitrogen) and Alexa Fluor 488 goat anti mouse IgG (1:1000, Invitrogen) or Alexa Fluor 488 goat anti mouse IgM (1:500, Invitrogen), at 37℃ for 1 hour. DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPI, Fluka). The results were registered with Leica SP8 confocal and processed by Adobe PhotoShop. **BiFC and Yeast two hybrid.** ORF of AtNTR1 and ILP1 were cloned to pSPYNE173 and pSPYCE(M) (Waadt *et al*, 2008), protoplast isolation and transformation were performed as described (Wu *et al*, 2009). Fluorescence images were acquired by Nikon Eclipse TE2000-E inverted microscope and processed by Nikon EZ-C1 software. For yeast two-hybrid assay, The AtNTR1 and ILP1 ORF were amplified from cDNA and fused in frame to pGBT9 Gal4 binding domain (BD) and pGAD424 Gal4 activating domain (AD) (Clontech). The yeast strain AH109 was co-transformed with corresponding vector and grown on dropout medium –LT (leucin and trypsin deficient) for selection at 28C. Serial decimal dilutions were used for low stringency selection on –LHT (leucin, trypsin and histidine) plates and high stringency selection on –LHTA (leucin, trypsin, histidine and adenine deficient) plates. **Splicing analysis.** Splicing analysis was performed using 6-FAM (Sigma Aldrich) labelled forward primer and capillary electrophoresis on ABI3730 DNA Analyzer (Life Technologies) as described (Raczynska *et al*, 2014; Simpson *et al*, 2007). Primer sequences for alternative spliced mRNA are given in Supplementary Table S7. Peak areas for each alternative variant were analysed using PeakScanner (Life Technologies) and contribution of each alternative isoforms was calculated as percentage of total isoforms detected. Means and standard deviations were calculated for three separate biological repetitions. Changes in AS events were considered significant if p-value<0.05 (t-test) and change ≥5%. AtNTR1 splicing has been analysed using alternative splicing panel (Ji *et al*, 2007). We therefore reanalysed a selected set of 144 alternative splicing events chosen to be robust in both 2 and 3 weeks old seedlings as described in figures legend. *DOG1* alternative splicing analysis shown in Supplementary Fig 1D was performed as described in (Schwab, 2008). **Consensus analysis** Splicing sites of affected and unaffected introns from our data set have been aligned and Web logo were created using Weblogo3 generator (Schneider and Stephens 1990; Crooks 2004). *Arabidopsis* consensus was created using introns from AATDB ver 3-5. For AtNTR1 dependent and independent intron analysis only introns with alternative 5` and 3` site selection where used. Statistical significance was calculated using Fisher two tail exact test based on frequencies of A/G A dinucleotides at positions +3 /+4 in changed and unchanged introns with alternative splice sites (Fisher 1922). **Chemical inhibition of splicing** **and elongation.** Plant were grown in liquid culture in 1/2MS with sucrose 15g/l, 2 weeks old plants where incubated with inhibitor for 3h before harvest. 6AU (10mg/l), MPA (5mg/l) or Herboxidiene (1.5mg/l) where used at final concentrations shown. For 6AU viability assay plants where grown on MS plates with 6AU (0.5mg/l). **Chromatin immunoprecipitation (ChIP).** Chromatin immunoprecipitation was performed as described (Bowler *et al*, 2004) with IP buffer prepared as described (Kaufmann *et al*, 2010). Chromatin was sonicated at 4 °C with a Diagenode Bioruptor at high intensity for 10 min (30s on/30s off). Antibodies: total PolII (Agrisera AS11 1804), P-Ser5 (Santa Cruz sc-47701) or peptide purified AtNTR1 antibodies described above were used with Dynabeads Protein G (Life Technologies). Chelex (Biorad) was used for de-crosslinking as described (Nelson *et al*, 2006). No antibody control was used to determine nonspecific background. Percentage of input was calculated for each sample using quantitive PCR. Primer sequences for qPCR are given in Supplementary Table S8. No antibody control showed signal an order of magnitude lower then performed side by side PolII ChIP experiment (Supplementary Figure S8 bottom panel). **RNA immunoprecipitation (RIP).** Nuclear fraction was purified as for ChIP. RNA specific steps were performed as described (Rowley *et al*, 2013). NTR1 complexes were immunoprecipitated using anti-GFP antibody (Chromotek gt-250). RNA was treated with DNase and reverse transcribed using random primers (Thermo Scientific) and SuperScriptIII RT (Life Technologies).

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**Figure 1**. AtNTR1 mutation results in reduced seed dormancy, low DOG1 expression, and a tendency towards downstream splice site selection. (**A**) Photographs and(**B**) quantification of seed dormancy tests. The chart represents the average percentage of germinated seeds without stratification after 4 days of growth in LD. The error bars represent ±SE (*n*=3). Tests were performed on freshly harvested seeds, with or without 3 days of stratification growth in LD. (**C**) The qPCR of *DOG1* expression in siliques (16 days after pollination). The graph represents the average ratio of *DOG1* to *UBC*, normalised to Col-0 (WT). The error bars represent ±SE (*n*=3). (**D**) *DOG1* splicing was assessed by RT-PCR combined with capillary electrophoresis. The graph represents the mean relative contribution of the mRNA forms found in the total pool of amplified products. The black and grey bars represent the data for Col-0 and *atntr1-1*, respectively. The error bars represent ±SD (*n*=3). To the right of the charts, the structures of the examined transcripts are shown (black boxes - constitutive exons, white boxes - alternative regions, black lines - introns). The black arrows show the locations of primers. (**E**) Directionality of splice site selection in *atntr1.* Splicing was analysedin 14 days old MS grown plants. For each type of alternative splice event, the black and white boxes show the contributions of opposite direction splicing events. The numbers represent the percentage of splice events supporting the direction of the splice site event change (also shown on horizontal axis). The numbers on the right hand panel represent the number of affected splicing events versus total number of splicing events analysed. The white bars represent distal 3’ and downstream 5’ splice site selection (3’SS/5’SS), exon skipping (ES), and intron retention (IR), while the black bars represent proximal 3’ and upstream 5’ splice site selection (3’SS/5’SS), exon inclusion (ES), and intron splicing (IR).

**Figure 2.** AtNTR1 interacts with U6 and U1 snRNA and co-localise with PolII. (**A**) Electrophoresis of RT-PCR products showing interactions of AtNTR1 with selected snRNA targets detected by RIP. The level of transcripts co-precipitated from transgenic plants expressing ANTR1-GFP (IP+) or wild-type plants (IP-) using anti-GFP antibody was measured by RT-PCR normalised to the inputs. To control for amplification from gDNA, controls without reverse transcriptase (RT-) were performed. U3 snRNA and 18srRNA were used as a negative control for interaction. (**B**) Fluorescent immunostaining of nuclei showing the co-localisation of AtNTR1 with SC35, total PolII, Ser5-phosphorylated PolII (PolIIA), or Ser2-phosphorylated PolII (PolIIO). AtNTR1 was detected using an antibody raised against ATNTR1 peptide.

**Figure 3.** AtNTR1 is present at *DOG1* gene. AtNTR1 antibodies were used to analyse AtNTR1 protein presence at *DOG1* locus using ChIP. Data shown represent enrichment above background level measured in *atntr1-1* mutant. Gene structure is shown with black boxes representing constitutive exons, gray boxes - alternative region, white boxes - promoter region, black lines – introns. Red lines show amplified regions. 0.5 kb scale is shown. Error bars represent ± SD of three independent experiments. As an additional negative control primers amplifying an unlinked intergenic region (IGR) were used.

**Figure 4.** *atntr1* in contrast to Herboxidiene treated plants show localised decrease in PolII occupancy on alternatively spliced exons. Line charts present ChIP profile of total PolII on examined genes. Black, grey and dashed grey lines represent results for Col-0, *atntr1-1* and Herboxidiene treated WT plants respectively. Above each chart, gene structure is shown with black boxes representing constitutive exons, gray boxes - alternative regions, white boxes - promoter region, black lines – introns. Red lines show amplified regions. Above each gene structure 0.5 kb scale is shown. Arrows on gene structure show localization of primers used for splicing analysis by RT-PCR, which was followed by capillary electrophoresis (results shown in tables next to each gene). Tables represent splicing site selection in mutant and Herboxidiene treated plants in comparison to wild type. For each chart the mean value from three independent experiments is shown. Error bars represent ± SD, \*\* indicates p<0.01 and \* p<0.05 of t-test.

**Figure 5.** Strong alternative splice sites constitute an AtNTR1 dependent transcriptional elongation pause site for PolII. Transgenic plants expressing 5`SS wt and 5`SS strong constructs were used to perform ChIP experiments using total PolII antibodies. Reporter gene structure is shown with black boxes representing constitutive exons, gray boxes - alternative region, white boxes - promoter region, black lines – introns. Red lines show amplified regions. Above gene structure a 0.5 kb scale is shown. Arrows on gene structure show localization of primers used for splicing analysis. Error bars represent ± SD of three independent transgenic lines, \*\* indicates p<0.01 and \* p<0.05 of t-test. Bottom panel shows a schematic representation of 5`SS strong – NTR1 dependent and 5`SS wt – NTR1 independent consensus as described in methods.

**Figure 6.** TFIISmut shows splicing defects consistent with kinetic coupling of transcription and splicing. (**A**) TFIISmut shows upstream splice site selection. The splicing was assessed by RT-PCR and capillary electrophoresis in 3 week old soil grown plants. The chart represents the average relative contribution of the mRNA forms found in the total pool of amplified products. The error bars represent ±SD (n=3). To the right of the charts, the structures of the examined transcripts are shown. The black boxes, white boxes, and black lines represent constitutive exons, alternative regions, and introns, respectively. The black arrows show the locations of the primers. Representative splicing assays are shown. (**B**) Directionality of splice site selection in TFIISmut. For each type of alternative splice event, the black and white boxes show the contributions of opposite direction splicing events. The numbers represent the percentage of splice events supporting the direction of the splice site event change as also shown on horizontal axis. The numbers on the right hand panel represent the number of affected splicing events versus total number of splicing events analysed. The white bars represent distal 3’ and 5’ splice site selection (3’SS/5’SS), exon skipping (ES), and intron retention (IR), while the black bars represent upstream 3`/5’ splice site selection (3’SS/5’SS), exon inclusion (ES), and intron splicing (IR). (**C**) TFIISmut and the *atntr1* mutant have opposite splicing phenotypes. Heat map with scale representing the absolute difference between each respective mutant and Col-0 in alternative splice site usage. The colours represent the direction of the events as described on the axis. Each splicing event is labelled with the gene name and the type of alternative splicing event. The 3’SS/5’SS notation signifies upstream/downstream 5’/3` splice site selection; ES marks exon skipping or exon inclusion; IR represents intron retention or intron splicing.

Table **I** AtNTR1 co-purifying proteins

Seedlings of complementing AtNTR1-GFP transgenic lines, expressed under native promoter, were used for four independent purifications with three negative controls (Col-0). After trypsin digestion and mass spectrometry, proteins identified in all purifications but not in negative controls were listed in the table. Number of unique peptides matching each identified protein was shown separately for each purification (P1-4).

**Supplementary Figure S1.** AtNTR1 has pleiotropic phenotypes. (**A**) Chart shows average percentage of germinated seeds at indicated time after sowing. Error bars represent ±SE (*n*=3). Freshly harvested seeds were sown directly and grown in LD, germination was assessed daily. *atntr1* has weak seed dormancy. (**B**) *atntr1* show lower *DOG1* expression in seedlings. Chart represent results of quantitive RT-PCR analysis performed on 14 days old plants grown in LD. The graph represents the average *DOG1* to *UBC* ratio normalised to Col-0. Error bars represent ±SE (*n*=3). (**C**) Schematic representation of DOG1 gene and alternatively spliced isoforms. White boxes represent exons; black lines represent introns; black boxes and checked boxes represent alternative regions. (**D**) Average relative contribution of DOG1 alterative isoforms measured by quantitive RT-PCR analysis on 14 days old plants grown in LD. Black and grey bars represent results for Col-0 and *atntr1-1* respectively. Error bars represent ±SD (*n*=3). (**E**) The splicing was assessed by RT-PCR and capillary electrophoresis. The chart represents the average relative contribution of the mRNA forms found in the total pool of amplified products. The error bars represent ±SD (n=3). To the right of the charts, the structures of the examined transcripts are shown. The black boxes, white boxes, and black lines represent constitutive exons, alternative regions, and introns, respectively. The black arrows show the locations of the primers. Representative splicing assays are shown. (**F**) Pictures taken at bolting time for *atntr1. atntr1* mutation in *Arabidopsis* results in late flowering. Plants were grown in LD conditions in soil. (**G**) Chart shows results of quantitive RT-PCR analysis on 14 days old plants grown in LD. The graph represents the average *FLC* to *UBC* ratio normalised to Col-0. *atntr1* shows high *FLC* expression in seedlings. Error bars represent ±SE (*n*=3). (**H**) *atntr1* mutant show a change in leaf morphology with shorter, round leaf blades. Plants were grown in LD conditions in soil, at bolting time leafs were detached and picture taken. White bars represent 5 mm. (**I**) *atntr1* shows lethality when grown at elevated temperature. WT Col-0, *atntr1-1*, *atntr1-1* complemented with WT copy of *AtNTR1* (AtNTR1) or *atntr1-1* complemented with GFP fused copy of *AtNTR1* (AtNTR1-GFP) were grown at 22 ºC and 30ºC on plates in LD. White bar represents 3 mm. (**J**) Complementing AtNTR1-GFP lines were used for analysis of NTR1 intracellular localization. Plants where grown on plates and root tip were visualized using fluorescent microscopy and GFP specific filters or white field (WF). White bar represent 50 µm.

**Supplementary Figure S2.** AtNTR1 is required for efficient splicing of strong alternative splice sites. WebLogo generated from *Arabidopsis* whole genome intron consensus (top panel), introns showing splicing defects in NTR1 (middle panel) and NTR1 independent introns (bottom panel) for donor (**A**) and acceptor (**B**) splice sites. X-axes correspond to nucleotides surrounding donor and acceptor splice site, position +1 correspond to first nucleotide after the exon-intron (**A**) and intron-exon (**B**) border. Y-axes represent information contents in alignment and nucleotide frequency, error bars represent Bayesian 95% confidence interval as described in (Crooks, 2004). (**C**) Structure and sequence changes introduced into reporter construct used to recreate an AtNTR1 splicing consensus. Line labelled 5`SS wt show sequence cloned from At1g23970 gene. Line labelled consensus show AtNTR1 target consensus sequence. Line labelled 5`SS strong shows sequence in construct mutagenised to resemble AtNTR1 target with changed nucleotides highlight in red. (**D**) Contribution of alternative isoforms in WT and *atntr1* plants for 5`SS wt (top panel) and 5`SS strong constructs (bottom panel) in transiently transformed *A. thaliana*  plants \*\* indicates p<0.01 of t-test.

**Supplementary Figure S3.** AtNTR1 interacts directly with ILP1 and *ilp1* shows similar phenotypes to *atntr1*. (**A**) *ilp1* show a similar phenotypic defects to *antr1* including lethality at elevated temperature and late flowering. WT Col-0, *atntr1-1*, *atntr1-2*, *ilp1-1*, *ilp1-2*, *atntr1-1* complemented with WT copy of *AtNTR1* (AtNTR1) or *atntr1-1* complemented with *GFP* fused copy of *AtNTR1* (AtNTR1-GFP) were grown either at 22 ºC and 30 ºC on plates in LD or in soil. White bars represent 5 mm. (**B**) Visualisation of the ILP1–AtNTR1 interaction by BiFC. The C-terminal (YFPc) and N-terminal (YFPn) parts of YFP were fused to AtNTR1 and ILP, respectively. Whole YFP was used as a positive control. The fusion protein and partial YFP were negative controls. (Chl: chlorophyll, WF: white field). (**C**) ILP1 and AtNTR1 interacted in a yeast two-hybrid assay. The yeast strain AH109 was co-transfected with ILP1 and AtNTR1 fused with either Gal4 DNA-binding domain (BD) or activating domain (AD) and grown on leucine- and trypsin-deficient medium (-LT) to select for transformants; on leucine-, trypsin-, and histidine-deficient medium (-LHT) to select for weak interactors; or on leucine-, trypsin, histidine-, and adenine-deficient medium (-LHTA) to select for strong interactors. (**D**) The *ilp1-1* mutant shows similar splicing defects to *atntr1*. Splicing was assessed by RT-PCR combined with capillary electrophoresis. The graph represents the average relative contribution of the mRNA forms found in the total pool of amplified products. The error bars represent ±SD (n=3). To the right of the charts, the structures of the examined transcript are shown. The black boxes, white boxes, and black lines represent constitutive exons, alternative regions, and introns, respectively. The black arrows show the locations of primers. Representative splicing assays are shown.

**Supplementary Figure S4.** AtNTR1 is present at selected targets. AtNTR1 antibodies were used to analyse AtNTR1 protein presence at selected targetsusing ChIP. Data shown represent enrichment above background level measured in *atntr1-1* mutant. Gene structure is shown with black boxes representing constitutive exons, gray boxes - alternative region, white boxes - promoter region, black lines – introns. Red lines show amplified regions. 0.5 kb scale is shown. Error bars represent ± SD of three independent experiments.

**Supplementary Figure S5.** *atntr1* plants but not Herboxidiene treated plants show localised decrease in PolII occupancy on alternatively spliced exons. Line charts present ChIP profile of total PolII on examined genes. Black, grey and dashed grey lines represent results for Col-0, *atntr1-1* and Herboxidiene treated WT plants respectively. Above each chart, gene structure is shown with black boxes representing constitutive exons, gray boxes - alternative regions, white boxes - promoter region, black lines – introns. Red lines show amplified regions. Above each gene structure 0.5 kb scale is shown. Arrows on gene structure show localization of primers used for splicing analysis by RT-PCR and capillary electrophoresis, shown in tables next to each gene. Tables represent splicing site selection in mutant and Herboxidiene treated plants in comparison to wild type. For each chart the mean value from three independent experiments is shown. Error bars represent ± SD, \*\* indicates p<0.01 and \* p<0.05 of t-test.

**Supplementary Figure S6.** *atntr1* plants but not *sr45-1* or *smD3-b* show localised decrease in PolII occupancy on alternatively spliced exons. Line charts present ChIP profile of total PolII on examined genes. Black, grey dashed black and dashed grey lines represent results for Col-0, *atntr1-1*, *sr45-1* and *smD3-b* plants respectively. Above each chart, gene structure is shown with black boxes representing constitutive exons, gray boxes - alternative regions, white boxes - promoter region, black lines – introns. Red lines show amplified regions. Above each gene structure 0.5 kb scale is shown. Arrows on gene structure show localization of primers used for splicing analysis by RT-PCR and capillary electrophoresis, shown in tables next to each gene. Tables represent splicing site selection in mutants in comparison to wild type. For each chart the mean value from three independent experiments is shown. Error bars represent ± SD, \*\* indicates p<0.01 and \* p<0.05 of t-test.

**Supplementary Figure S7.** TFIISmut shows strong developmental phenotypes and deregulation of alternative splicing. (**A**) DNA sequences of conserved ADEP amino acid sequence (upper panel) and its mutation into AAAP (lower panel) shown for *TFIIS* coding constructs. (**B**) Pictures taken at bolting of TFIISmut plant showing WT Col-0, *tfIIs-1*, and TFIISmut plants. Plants were grown in soil in LD conditions. (**C**) Splicing defects in TFIISmut. Splicing was assessed by RT-PCR and capillary electrophoresis. Graph represents relative contribution of mRNA forms found in total pool of amplified products. Black and grey bars show data for Col-0 and TFIISmut respectively. Error bars represent ±SD (*n*=3). Next to chart, structure of examined transcript is shown. Black boxes represent constitutive exons, white boxes - alternative regions and black lines - introns. Black arrows show localization of primers. (**D**) All splicing events changed in *tfIIs-1* (10 out of 284) are also changed in TFIISmut (10+52 out of 284).

**Supplementary Figure S8.** *atntr1* and TFIISmut show opposite changes in PolII occupancy on alternatively spliced exons. Line charts present profile of total RNA PolII on examined genes. Black, grey and dashed grey lines represent results for Col-0, TFIISmut and *atntr1-1* respectively. Above each chart, gene structure is shown with black boxes representing constitutive exons, gray boxes - alternative regions, white boxes - promoter region, black lines – introns. Red lines show amplified regions. Above each gene structure 0.5 kb scale is shown. Bar graphs represent ChIP levels of P-Ser5 RNA PolII for alternatively spliced regions. Arrows on gene structure show localization of primers used for splicing analysis by RT-PCR and capillary electrophoresis, which is shown in tables. Tables represent splicing site selection in both mutants in comparison to wild type. For each chart the mean value from three independent experiments is shown. Error bars represent ± SD, \*\* indicates p<0.01 and \* p<0.05 of t-test. Bottom panel shows results from a no antibody control for *DOG1* performed in WT plants side by side with the above experiments.

**Supplementary Figure S9.** *atntr1*TFIISmut double mutant show partial reversal of *atntr1* splicing defects. (**A**) Splicing analysis in double mutant of *atntr1-1*TFIISmut, *atntr1-1*, TFIISmut and WT plants. The splicing was assessed by RT-PCR and capillary electrophoresis. The chart represents the average relative contribution of the mRNA forms found in the total pool of amplified products. The error bars represent ±SD (n=3). To the right of the charts, the structures of the examined transcripts are shown. The black boxes, white boxes, and black lines represent constitutive exons, alternative regions, and introns, respectively. The black arrows show the locations of the primers. Representative splicing assays are shown. (**B**) Heat map representation of splicing defects caused by TFIISmut in WT and in *atntr1* background. The colours represent the splicing isoforms change between TFIISmut and WT first column and between TFIISmut and *atntr1-1*TFIISmut second column. Only events with significant change detected between *atntr1* and WT were included. \* indicate p<0.05 of t-test and change ≥5% between *atntr1*TFIISmut and *atntr1*. Scale represents the absolute difference between each respective mutant and Col-0 in alternative splice site usage. Each splicing event is labelled with the gene name and the type of alternative splicing event. The downstr. and upstr. notation signifies downstream or upstream 3` and 5’ splice site selection; ES marks exon skipping or exon inclusion; IR represents intron retention or intron splicing. (**C**) TFIISmut transgene is expressed at similar level in WT and *atntr1-1* plants.*atntr1-1* mutants were transformed by TFIISmut transgene. 3 independent transformants were recovered and analyzed by qRT-PCR for the expression of TFIISmut transgene. Error bars represent ± SD (n=3).

**Supplementary Figure S10.** 6AUtreated *atntr1* mutant show partial reversal of *atntr1* splicing defects. (**A**) Splicing analysis in WT, *atntr1-1* andWT treated with 6AU or MPA. The splicing was assessed by RT-PCR and capillary electrophoresis. The chart represents the average relative contribution of the mRNA forms found in the total pool of amplified products. The error bars represent ±SD (n=3). To the right of the charts, the structures of the examined transcripts are shown. The black boxes, white boxes, and black lines represent constitutive exons, alternative regions, and introns, respectively. The black arrows show the locations of the primers. Representative splicing assays are shown. (**B**) Heat map representation of 6AU mediated reverses the *atntr1* splicing defects. The colours represent the splicing isoforms change between *atntr1-1* and WT first column and between *atntr1-1*6AU and WT second column or *atntr1-1*6AU and *atntr1-1*. \* indicate p<0.05 of t-test. Scale represents the absolute difference between each respective mutant and Col-0 in alternative splice site usage. Each splicing event is labelled with the gene name and the type of alternative splicing event. The 5’SS and 3’SS signifies alternative 5’ and 3’ splice site selection respectively; ES marks exon skipping or exon inclusion; IR represents intron retention or intron splicing.

**Supplementary Figure S11.** *atntr1* mutant is less sensitive then WT to grow inhibition by 6AU. WT, *atntr1-1*, *atntr1-2* and *tfIIs-1* plants wheregrown on MS or MS supplemented with 6AU for number of days shown. Bar represent 5mm scale.

**Table I**  AtNTR1 co-purifying proteins

|  |  |  |  |
| --- | --- | --- | --- |
| Gene ID | Gene name | MW(Da) | Number of  unique peptides  P1-P2-P3-P4 |
| AT1G17070 | AtNTR1 | 96937 | 37-36-26-30 |
| AT5G08550 | ILP1 | 100998 | 21-22-30-28 |
| AT1G24180 | IAR4 | 43787 | 1-2-2-2 |
| AT2G39770 | CYT1 | 39837 | 1-1-1-2 |
| AT2G47580 | U1A | 58456 | 1-1-1-1 |
| AT2G30050 | WD40 | 32907 | 1-1-1-1 |

Agafonov DE, Deckert J, Wolf E, Odenwälder P, Bessonov S, Will CL, Urlaub H & Lührmann R (2011) Semiquantitative proteomic analysis of the human spliceosome via a novel two-dimensional gel electrophoresis method. *Mol. Cell. Biol.* **31:** 2667–2682

Alexander RD, Innocente SA, Barrass JD & Beggs JD (2010) Splicing-dependent RNA polymerase pausing in yeast. *Mol. Cell* **40:** 582–593

Batsché E, Yaniv M & Muchardt C (2005) The human SWI/SNF subunit Brm is a regulator of alternative splicing. *Nat. Struct. 38 Mol. Biol.* **13:** 22–29

Bentsink L, Jowett J, Hanhart CJ & Koornneef M (2006) Cloning of DOG1, a quantitative trait locus controlling seed dormancy in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* **103:** 17042–17047

Boon K-L, Auchynnikava T, Edwalds-Gilbert G, Barrass JD, Droop AP, Dez C & Beggs JD (2006) Yeast Ntr1/Spp382 Mediates Prp43 Function in Postspliceosomes. *Mol. Cell. Biol.* **26:** 6016–6023

Bowler C, Benvenuto G, Laflamme P, Molino D, Probst AV, Tariq M & Paszkowski J (2004) Chromatin techniques for plant cells. *Plant J.* **39:** 776–789

Braberg H, Jin H, Moehle EA, Chan YA, Wang S, Shales M, Benschop JJ, Morris JH, Qiu C, Hu F, Tang LK, Fraser JS, Holstege FCP, Hieter P, Guthrie C, Kaplan CD & Krogan NJ (2013) From Structure to Systems: High-Resolution, Quantitative Genetic Analysis of RNA Polymerase II. *Cell* **154:** 775–788

Brody Y, Neufeld N, Bieberstein N, Causse SZ, Böhnlein E-M, Neugebauer KM, Darzacq X & Shav-Tal Y (2011) The In Vivo Kinetics of RNA Polymerase II Elongation during Co-Transcriptional Splicing. *PLoS Biol.* **9:** e1000573

Chanarat S, Seizl M & Strässer K (2011) The Prp19 complex is a novel transcription elongation factor required for TREX occupancy at transcribed genes. *Genes Dev.* **25:** 1147–1158

Chathoth KT, Barrass JD, Webb S & Beggs JD (2014) A splicing-dependent transcriptional checkpoint associated with prespliceosome formation. *Mol. Cell* **53:** 779–790

Cheung ACM & Cramer P (2011) Structural basis of RNA polymerase II backtracking, arrest and reactivation. *Nature* **471:** 249–253

Close P, East P, Dirac-Svejstrup AB, Hartmann H, Heron M, Maslen S, Chariot A, Söding J, Skehel M & Svejstrup JQ (2012) DBIRD complex integrates alternative mRNA splicing with RNA polymerase II transcript elongation. *Nature* **484:** 386–389

Crooks GE (2004) WebLogo: A Sequence Logo Generator. *Genome Res.* **14:** 1188–1190

Cvitkovic I & Jurica MS (2013) Spliceosome database: a tool for tracking components of the spliceosome. *Nucleic Acids Res.* **41:** D132–141

Diao Y, Guo X, Li Y, Sun K, Lu L, Jiang L, Fu X, Zhu H, Sun H, Wang H & Wu Z (2012) Pax3/7BP Is a Pax7- and Pax3-Binding Protein that Regulates the Proliferation of Muscle Precursor Cells by an Epigenetic Mechanism. *Cell Stem Cell* **11:** 231–241

Dujardin G, Lafaille C, de la Mata M, Marasco LE, Muñoz MJ, Le Jossic-Corcos C, Corcos L & Kornblihtt AR (2014) How Slow RNA Polymerase II Elongation Favors Alternative Exon Skipping. *Mol. Cell* **54:** 683–690

Dutertre M, Sanchez G, De Cian M-C, Barbier J, Dardenne E, Gratadou L, Dujardin G, Le Jossic-Corcos C, Corcos L & Auboeuf D (2010) Cotranscriptional exon skipping in the genotoxic stress response. *Nat. Struct. Mol. Biol.* **17:** 1358–1366

Exinger F & Lacroute F (1992) 6-Azauracil inhibition of GTP biosynthesis in Saccharomyces cerevisiae. *Curr. Genet.* **22:** 9–11

Filichkin SA, Priest HD, Givan SA, Shen R, Bryant DW, Fox SE, Wong W-K & Mockler TC (2009) Genome-wide mapping of alternative splicing in Arabidopsis thaliana. *Genome Res.* **20:** 45–58

Grasser M, Kane CM, Merkle T, Melzer M, Emmersen J & Grasser KD (2009) Transcript elongation factor TFIIS is involved in arabidopsis seed dormancy. *J. Mol. Biol.* **386:** 598–611

Hasegawa M, Miura T, Kuzuya K, Inoue A, Won Ki S, Horinouchi S, Yoshida T, Kunoh T, Koseki K, Mino K, Sasaki R, Yoshida M & Mizukami T (2011) Identification of SAP155 as the Target of GEX1A (Herboxidiene), an Antitumor Natural Product. *ACS Chem. Biol.* **6:** 229–233

Ho SN, Hunt HD, Horton RM, Pullen JK & Pease LR (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77:** 51–59

Howe KJ, Kane CM & Ares M Jr (2003) Perturbation of transcription elongation influences the fidelity of internal exon inclusion in Saccharomyces cerevisiae. *RNA N. Y. N* **9:** 993–1006

Ip JY, Schmidt D, Pan Q, Ramani AK, Fraser AG, Odom DT & Blencowe BJ (2011) Global impact of RNA polymerase II elongation inhibition on alternative splicing regulation. *Genome Res.* **21:** 390–401

Izban MG & Luse DS (1992) The RNA polymerase II ternary complex cleaves the nascent transcript in a 3’----5’ direction in the presence of elongation factor SII. *Genes Dev.* **6:** 1342–1356

Ji Q, Huang C-H, Peng J, Hashmi S, Ye T & Chen Y (2007) Characterization of STIP, a multi-domain nuclear protein, highly conserved in metazoans, and essential for embryogenesis in Caenorhabditis elegans. *Exp. Cell Res.* **313:** 1460–1472

Jones MA, Williams BA, McNicol J, Simpson CG, Brown JWS & Harmer SL (2012) Mutation of Arabidopsis SPLICEOSOMAL TIMEKEEPER LOCUS1 Causes Circadian Clock Defects. *Plant Cell* Available at: http://www.plantcell.org/cgi/doi/10.1105/tpc.112.104828 [Accessed November 13, 2012]

Kaida D, Motoyoshi H, Tashiro E, Nojima T, Hagiwara M, Ishigami K, Watanabe H, Kitahara T, Yoshida T, Nakajima H, Tani T, Horinouchi S & Yoshida M (2007) Spliceostatin A targets SF3b and inhibits both splicing and nuclear retention of pre-mRNA. *Nat. Chem. Biol.* **3:** 576–583

Kaufmann K, Muiño JM, Østerås M, Farinelli L, Krajewski P & Angenent GC (2010) Chromatin immunoprecipitation (ChIP) of plant transcription factors followed by sequencing (ChIP-SEQ) or hybridization to whole genome arrays (ChIP-CHIP). *Nat. Protoc.* **5:** 457–472

Konforti BB, Koziolkiewicz MJ & Konarska MM (1993) Disruption of base pairing between the 5′ splice site and the 5′ end of U1 snRNA is required for spliceosome assembly. *Cell* **75:** 863–873

Koodathingal P, Novak T, Piccirilli JA & Staley JP (2010) The DEAH Box ATPases Prp16 and Prp43 Cooperate to Proofread 5′ Splice Site Cleavage during Pre-mRNA Splicing. *Mol. Cell* **39:** 385–395

Lagisetti C, Yermolina MV, Sharma LK, Palacios G, Prigaro BJ & Webb TR (2014) Pre-mRNA splicing-modulatory pharmacophores: the total synthesis of herboxidiene, a pladienolide-herboxidiene hybrid analog and related derivatives. *ACS Chem. Biol.* **9:** 643–648

Liu Y, Koornneef M & Soppe WJJ (2007) The absence of histone H2B monoubiquitination in the Arabidopsis hub1 (rdo4) mutant reveals a role for chromatin remodeling in seed dormancy. *Plant Cell* **19:** 433–444

Logemann E, Birkenbihl RP, Ulker B & Somssich IE (2006) An improved method for preparing Agrobacterium cells that simplifies the Arabidopsis transformation protocol. *Plant Methods* **2:** 16

Marquez Y, Brown JWS, Simpson C, Barta A & Kalyna M (2012) Transcriptome survey reveals increased complexity of the alternative splicing landscape in Arabidopsis. *Genome Res.* **22:** 1184–1195

De la Mata M, Alonso CR, Kadener S, Fededa JP, Blaustein M, Pelisch F, Cramer P, Bentley D & Kornblihtt AR (2003) A slow RNA polymerase II affects alternative splicing in vivo. *Mol. Cell* **12:** 525–532

De la Mata M, Muñoz MJ, Alló M, Fededa JP, Schor IE & Kornblihtt AR (2011) RNA Polymerase II Elongation at the Crossroads of Transcription and Alternative Splicing. *Genet. Res. Int.* **2011:** 1–9

Mayas RM, Maita H, Semlow DR & Staley JP (2010) Spliceosome discards intermediates via the DEAH box ATPase Prp43p. *Proc. Natl. Acad. Sci.* **107:** 10020

Miller-Wideman M, Makkar N, Tran M, Isaac B, Biest N & Stonard R (1992) Herboxidiene, a new herbicidal substance from Streptomyces chromofuscus A7847. Taxonomy, fermentation, isolation, physico-chemical and biological properties. *J. Antibiot. (Tokyo)* **45:** 914–921

Mortensen SA & Grasser KD (2014) The seed dormancy defect of Arabidopsis mutants lacking the transcript elongation factor TFIIS is caused by reduced expression of the DOG1 gene. *FEBS Lett.* **588:** 47–51

Nelson JD, Denisenko O & Bomsztyk K (2006) Protocol for the fast chromatin immunoprecipitation (ChIP) method. *Nat. Protoc.* **1:** 179–185

Pagani F, Stuani C, Zuccato E, Kornblihtt AR & Baralle FE (2003) Promoter architecture modulates CFTR exon 9 skipping. *J. Biol. Chem.* **278:** 1511–1517

Palusa SG, Ali GS & Reddy ASN (2007) Alternative splicing of pre-mRNAs of Arabidopsis serine/arginine-rich proteins: regulation by hormones and stresses: Stress regulation of alternative splicing of SR genes. *Plant J.* **49:** 1091–1107

Pandit S, Lynn B & Rymond BC (2006) Inhibition of a spliceosome turnover pathway suppresses splicing defects. *Proc. Natl. Acad. Sci.* **103:** 13700–13705

Pandya-Jones A & Black DL (2009) Co-transcriptional splicing of constitutive and alternative exons. *RNA N. Y. N* **15:** 1896–1908

Pan Q, Shai O, Lee LJ, Frey BJ & Blencowe BJ (2008) Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.* **40:** 1413–1415

Raczynska KD, Stepien A, Kierzkowski D, Kalak M, Bajczyk M, McNicol J, Simpson CG, Szweykowska-Kulinska Z, Brown JWS & Jarmolowski A (2014) The SERRATE protein is involved in alternative splicing in Arabidopsis thaliana. *Nucleic Acids Res.* **42:** 1224–1244

Reines D (1992) Elongation factor-dependent transcript shortening by template-engaged RNA polymerase II. *J. Biol. Chem.* **267:** 3795–3800

Riles L, Shaw RJ, Johnston M & Reines D (2004) Large-scale screening of yeast mutants for sensitivity to the IMP dehydrogenase inhibitor 6-azauracil. *Yeast* **21:** 241–248

Rowley MJ, Böhmdorfer G & Wierzbicki AT (2013) Analysis of long non-coding RNAs produced by a specialized RNA polymerase in Arabidopsis thaliana. *Methods San Diego Calif* **63:** 160–169

Saint-André V, Batsché E, Rachez C & Muchardt C (2011) Histone H3 lysine 9 trimethylation and HP1γ favor inclusion of alternative exons. *Nat. Struct. Mol. Biol.* **18:** 337–344

Schwab M (2008) Identification of Novel Seed Dormancy Mutants in Arabidopsis thaliana and Molecular and Biochemical Characterization of the Seed Dormancy Gene DOG1.

Sekimizu K, Kobayashi N, Mizuno D & Natori S (1976) Purification of a factor from Ehrlich ascites tumor cells specifically stimulating RNA polymerase II. *Biochemistry (Mosc.)* **15:** 5064–5070

Shirzadegan M, Christie P & Seemann JR (1991) An efficient method for isolation of RNA from tissue cultured plant cells. *Nucleic Acids Res.* **19:** 6055

Shukla S, Kavak E, Gregory M, Imashimizu M, Shutinoski B, Kashlev M, Oberdoerffer P, Sandberg R & Oberdoerffer S (2011) CTCF-promoted RNA polymerase II pausing links DNA methylation to splicing. *Nature* **479:** 74–79

Sigurdsson S, Dirac-Svejstrup AB & Svejstrup JQ (2010) Evidence that Transcript Cleavage Is Essential for RNA Polymerase II Transcription and Cell Viability. *Mol. Cell* **38:** 202–210

Simpson CG, Fuller J, Maronova M, Kalyna M, Davidson D, McNicol J, Barta A & Brown JWS (2007) Monitoring changes in alternative precursor messenger RNA splicing in multiple gene transcripts. *Plant J.* **53:** 1035–1048

Swaraz AM, Park Y-D & Hur Y (2011) Knock-out mutations of Arabidopsis SmD3-b induce pleotropic phenotypes through altered transcript splicing. *Plant Sci. Int. J. Exp. Plant Biol.* **180:** 661–671

Tannukit S, Wen X, Wang H & Paine ML (2008) TFIP11, CCNL1 and EWSR1 Protein-protein Interactions, and Their Nuclear Localization. *Int. J. Mol. Sci.* **9:** 1504–1514

Tsai R-T (2005) Spliceosome disassembly catalyzed by Prp43 and its associated components Ntr1 and Ntr2. *Genes Dev.* **19:** 2991–3003

Waadt R, Schmidt LK, Lohse M, Hashimoto K, Bock R & Kudla J (2008) Multicolor bimolecular fluorescence complementation reveals simultaneous formation of alternative CBL/CIPK complexes in planta. *Plant J. Cell Mol. Biol.* **56:** 505–516

Wahl MC, Will CL & Lührmann R (2009) The Spliceosome: Design Principles of a Dynamic RNP Machine. *Cell* **136:** 701–718

Wen X, Lei Y-P, Zhou YL, Okamoto CT, Snead ML & Paine ML (2005) Structural organization and cellular localization of tuftelin-interacting protein 11 (TFIP11). *CMLS Cell. Mol. Life Sci.* **62:** 1038–1046

Wu F-H, Shen S-C, Lee L-Y, Lee S-H, Chan M-T & Lin C-S (2009) Tape-Arabidopsis Sandwich - a simpler Arabidopsis protoplast isolation method. *Plant Methods* **5:** 16

Yoshimoto R, Okawa K, Yoshida M, Ohno M & Kataoka N (2014) Identification of a novel component C2ORF3 in the lariat-intron complex: lack of C2ORF3 interferes with pre-mRNA splicing via intron turnover pathway. *Genes Cells* **19:** 78–87

Yoshizumi T, Tsumoto Y, Takiguchi T, Nagata N, Yamamoto YY, Kawashima M, Ichikawa T, Nakazawa M, Yamamoto N & Matsui M (2006) Increased level of polyploidy1, a conserved repressor of CYCLINA2 transcription, controls endoreduplication in Arabidopsis. *Plant Cell* **18:** 2452–2468

Zhang C-J, Zhou J-X, Liu J, Ma Z-Y, Zhang S-W, Dou K, Huang H-W, Cai T, Liu R, Zhu J-K & He X-J (2013) The splicing machinery promotes RNA-directed DNA methylation and transcriptional silencing in Arabidopsis. *EMBO J.* **32:** 1128–1140