Alternative Splicing Control by Polypyrimidine Tract-Binding Proteins from *Arabidopsis thaliana*

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Summary

Alternative splicing (AS) is emerging as an important component of gene regulation in plants and has the potential to massively expand the transcriptome, while its functional implications and regulation are not fully understood. The heterogeneous ribonucleoprotein family member polypyrimidine tract-binding protein (PTB) is known to be involved in the regulation of a concerted AS program in differentiating neurons in mammals, however, PTBs in plants were poorly investigated and many questions regarding their regulatory functions in AS remain to be elucidated. Three PTB genes are encoded in the genome of *Arabidopsis thaliana,* of which two are closely related and one has a comparable homology to human PTB as it has to the two other Arabidopsis PTBs. This work demonstrates that PTBs from Arabidopsis have a gene regulatory potential affecting splicing of their pre-mRNAs in auto- and cross-regulatory circuits as well as having a transcriptome-wide impact on splice site selection. Deep RNA sequencing of AtPTB misexpression lines resulted in the identification of 452 AtPTB-regulated AS events. Interestingly, only AtPTB1 and AtPTB2, representing the two close homologues, were shown to regulate global splicing patterns, whereas AtPTB3 seems not play an important role in this process. In addition, AtPTB-mediated AS is linked to nonsense-mediated decay (NMD) as many target transcripts have NMD eliciting features and the interlocked feedback loops of AtPTB expression includes AS-coupled NMD. Importantly, we established that AtPTB-dependent AS events are coupled to diverse biological processes and upon AtPTB misexpression, changes in phytochrome interacting factor 6 splice patterns coincided with altered rates of abscisic acid-dependent seed germination. Furthermore, we found changes in AS patterns and expression levels of key flowering regulators in an AtPTB1/2 level-dependent manner. In addition to their role in splicing regulation, PTBs are multifunctional proteins involved in various processes of mRNA metabolism and we were able to establish a splicing-independent function of AtPTBs, coinciding with the subcellular localization of AtPTB fluorescent fusion proteins in the cytoplasm, nucleus, and processing bodies. In conclusion, this work shows that AtPTBs regulate their own expression by a negative feedback inhibition and have a global role in AS regulation in Arabidopsis.

Zusammenfassung

Mit der zunehmenden Menge an Transkriptomdaten zeichnet sich alternatives Spleißen (AS) in Pflanzen als wichtiger Schritt der Genexpression ab und hat ein bedeutendes Potenzial die Diversität des Transkriptoms massiv zu erhöhen. Die zu der Familie der heterogenen nukleären Ribonukleoproteinfamilie gehörenden Polypyrimidin Trakt-bindenden Proteine (PTB) regulieren ein genau abgestimmtes alternatives Spleißprogramm im Zuge der Differenzierung von Neuronen in Säugern. Dahingegen wurden die PTBs in Pflanzen bisher kaum erforscht und die zentrale Frage bezüglich ihrer regulatorischen Funktion im AS sind ungeklärt. Im Genom von *Arabidopsis thaliana* sind drei PTB Homologe (AtPTBs) zu finden, von denen zwei sehr eng miteinander verwandt sind und das dritte PTB Protein eine ähnliche Homologie zu den beiden anderen pflanzlichen PTBs wie zum humanen PTB aufweist. Die vorliegende Arbeit zeigt, dass AtPTBs ihre eigene Expression über AS regulieren wobei eine Transkriptisoform entsteht, die für das Protein kodiert, und eine zweite Isoform, die ein vorzeitiges Stoppkodon beinhaltet. Das vorzeitige Stoppkodon markiert diese Transkripte für den mRNA Abbaumechanismus „Nonsense-mediated mRNA decay“ (NMD) und dadurch konnte bewiesen werden, dass eine Kopplung von AS und NMD in der Regulation der AtPTB Expression stattfindet. Durch die transkriptom weite Analyse von AtPTB-Misexpressionslinien konnte die globale Rolle von AtPTBs im Prozess des AS aufgedeckt und 452 AtPTB-abhängige alternative Spleiß-Ereignisse identifiziert werden. Interessanterweise trifft dies aber nur für die zwei nahe verwandten Proteine AtPTB1 und AtPTB2 zu; AtPTB3 hingegen hat keinen wesentlichen Einfluss auf das AS in Arabidopsis. Zusätzlich konnten wir eine Verknüpfung von AtPTB-reguliertem AS mit dem Prozess der Abscisinsäure-vermittelten Samenkeimung herstellen und zeigen, dass AS und die Expression von zentralen Blühregulatoren AtPTB-abhängig reguliert sind. In Übereinstimmung mit einer Rolle pflanzlicher PTBs in Spleiß-unabhängigen regulatorischen Prozessen steht die subzelluläre Lokalisierung von fluoreszierenden AtPTB-Fusionsproteinen, die im Zellkern, dem Cytoplasma und auch Prozessierungskörperchen (P-Bodies) zu finden sind.

1 Introduction

In the course of messenger RNA (mRNA) maturation, multiple regulatory steps occur, including the addition of the 5' cap, removal of non-coding sequences, and 3' end maturation. Of all these processes mentioned, splicing describes an important step that is indispensable for the generation of translation-competent mRNAs. On one hand, constitutive splicing plays a fundamental role in gene expression, whereas on the other hand alternative splicing can increase transcriptome and proteome complexity by the generation of distinct splicing variants from one precursor mRNA (pre-mRNA). The processes of AS, and alternative 3' end processing were shown to massively expand transcriptome diversity in animals (Mangone et al., 2010; Nilsen and Graveley, 2010) and plants (Reddy, 2007; Hunt, 2011; Syed et al., 2012).

1.1. Alternative splicing in humans and plants

It has been estimated that AS occurs in 95% of all intron-containing human genes (Pan et al., 2008), and recent transcriptome-wide splicing analyses revealed a wide extent of AS in plants as well (Filichkin et al., 2010; Lu et al., 2010; Zhang et al., 2010; Marquez et al., 2012). The use of high-throughput RNA sequencing (RNA-seq) techniques revealed that 42% - 61% of all multi-exon genes from *Arabidopsis thaliana* (Filichkin et al., 2010; Marquez et al., 2012) and 33% - 48% of all rice genes (Lu et al., 2010; Zhang et al., 2010) are affected by AS. Over the past several years, various studies have shown that AS occurs in many metazoan organisms regulating a great diversity of essential biological processes. However, the biological significance and the gene regulatory mechanisms of the majority of AS events are not well understood and remain to be elucidated. One example for the regulatory potential of AS is its coupling with the eukaryotic RNA surveillance mechanism nonsense-mediated decay (NMD), since many alternatively spliced transcripts contain premature termination codons (PTC) or long 3' untranslated regions (UTRs) and are therefore potential NMD substrates

(Chang et al., 2007; Nicholson et al., 2010). Interestingly, a previous analysis addressing the coupling of AS and NMD indicated that 43% and 36% of all AS events from *Arabidopsis* and rice, respectively, produce transcripts that are potential NMD targets (Wang and Brendel, 2006). This was further substantiated by a recent study showing that out of 270 selected *Arabidopsis* genes, 32% generated splicing variants with elevated levels in NMD mutants (Kalyna et al., 2012).

Among the most common types of AS are exon skipping, intron retention, and the selection of alternative 5' (alt. 5') and/or 3' (alt. 3') splice sites (Black, 2003; Reddy, 2007) (Figure 1). The decision of splice site usage is dependent on the splicing code, an interacting network of *trans*-acting splice factors and *cis*-regulatory sequence elements (Chen and Manley, 2009). *Cis* elements, such as exonic/intronic splicing enhancers (ESE/ISE) or silencers (ESS/ISS), can be found all over the pre-mRNA and function not only as binding platforms for spliceosomal components, but also for splicing regulatory proteins such as heterogeneous nuclear ribonucleoproteins (hnRNPs) and serine/arginine-rich (SR) proteins.

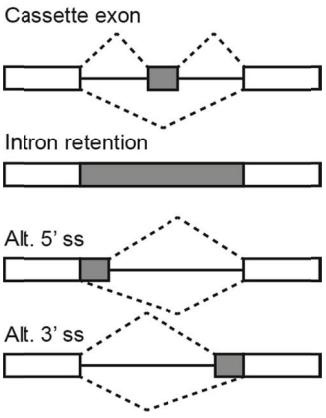

Figure 1: Common alternative splicing events. White boxes represent exons, gray boxes indicate the alternative spliced regions and lines display introns. The dashed line indicates the possible alternative splicing events. For the intron retention alternative splicing event, only the transcript variant with the retained intronic sequence is displayed. Alt. 5' ss, alternative 5' splice site; alt. 3' ss, alternative 3' splice site.

1.2. Alternative splicing is regulated by hnRNPs and SR proteins

Originally, hnRNPs were identified based on their binding to RNA polymerase II generated transcripts (Dreyfuss et al., 1993) and nowadays the collection of identified hnRNPs includes a diverse group of proteins that do not share a distinct structural or functional feature (Martinez-Contreras et al., 2007; Wachter et al., 2012). However, all hnRNPs can associate with RNA or

single stranded DNA and contain RNA recognition motifs (RRMs) or other functionally equivalent domains like KH domains or atypical RRMs (Martinez-Contreras et al., 2007). Apart from being involved in the regulation of splicing, hnRNPs have multiple functions and are associated with nearly every step of nucleic acid processing and/or maintenance in the nucleus as well as in the cytoplasm, such as chromatin remodelling, control of mRNA stability and modifications, and mRNA transport (Martinez-Contreras et al., 2007; Wachter et al., 2012). It is evident that hnRNPs have specific and overlapping functions but the processes under control are often dependent on a dynamic and co-operative action of various hnRNPs and other splicing factors, like SR proteins (Wachter et al., 2012) (Figure 2). This is also exemplified by the fact that more than 200 RNA binding proteins exist in Arabidopsis (Lorkovic, 2009) but the exact definition of the plant splicing code remains to be elucidated and future work will help to identify and verify the multiple regulatory layers of *trans*-acting splice factors.

So far, hnRNPs are best studied in the mammalian system but few reports on the regulatory function of plant hnRNPs in splicing reactions are available. The best studied plant hnRNP-like proteins are the glycine-rich RNA binding proteins (GRPs) AtGRP7 and AtGRP8 that have been shown to regulate their own expression in an auto- and cross-regulatory circuit (Staiger et al., 2003; Schoning et al., 2008). In addition to that, a recent work addressed the regulatory potential of AtGRP7 in AS and a direct interaction of AtGRP7 with its target pre-mRNAs was demonstrated by RNA immunoprecipitation (Streitner et al., 2012). Therefore, AtGRP7 is the first plant hnRNP with direct implications in a more global regulation of AS (Streitner et al., 2012) and it can be anticipated that hnRNPs are involved in the widespread regulation of AS shown by the transcriptome-wide analysis of polypyrimidine-tract binding proteins (PTBs) performed in our group (Rühl et al., 2012). PTBs are well investigated splicing regulatory proteins in the mammalian system and will be addressed in the next paragraph in more detail.

SR proteins are typically known to act antagonistically to hnRNPs and these evolutionary conserved splicing factors have a huge impact on the splicing outcome. One example is the regulation of the *Dscam* exon 6 cluster from

Drosophila melanogaster where the hnRNP HRP36 acts antagonistically to SR proteins to prevent the inclusion of multiple exons (Olson et al., 2007). SR proteins classically contain one or two N-terminal RRMs and a C-terminal domain rich in serine/arginine dipeptides that is responsible for protein-protein interactions (Reddy, 2004; Barta et al., 2010). In animals, it was shown that SR proteins can act as activators as well repressors of constitutive and alternative splicing reactions (Long and Caceres, 2009). Interestingly, the number of SR proteins varies between different groups of organisms. Whereas the human genome encodes 12 SR proteins, 18 and 22 can be found in Arabidopsis and rice, respectively (Reddy and Shad Ali, 2011). Among the plant SR proteins are orthologues of the mammalian system as well as plant-specific SR proteins (Reddy and Shad Ali, 2011), which might be explained by plant-specific *cis*-elements and differences in intron/exon architecture. The relative length of introns and exons varies across the eukaryotic kingdom and it is important to note that splicing in plants seems to predominantly depend on an intron definition model (Reddy et al., 2012) which is in contrast to the model of prevalent exon definition found in mammals (De Conti et al., 2012).

Typically, binding sites of hnRNPs and SR protein are present within the vicinity of exon/intron junctions, suggesting that the interplay between activation and repression modulates the probability of the AS event under control. The classical view was that hnRNPs and SR proteins act as repressors and activators of splicing reactions, respectively, but it is now becoming evident that the mode of action of splicing regulatory proteins often is context-dependent. Each exon is flanked by a unique pair of splice site signals and additional *cis*-regulatory elements like ISE/ISI and ESE/ESI. At the end, the sum of contributions from SR proteins, hnRNPs, and additional regulatory factors defines the splicing outcome and can vary dependent on their binding position within the pre-mRNA (Busch and Hertel, 2012; Wachter et al., 2012) (Figure 2).

1.3. Polypyrimidine-tract binding proteins

A well-studied member of the hnRNP family is PTB, representing an important trans-acting factor interfering with splice site recognition. PTB has been extensively characterized to bind in a sequence-specific manner to CU-rich regulatory elements in RNA targets near PTB-repressed exons and thereby alters spliceosome assembly at adjacent splice sites (Spellman and Smith, 2006). CU-rich elements are also found between the branch point and 3' splice sites in most constitutively spliced genes providing a binding platform for essential splicing factors like $U2AF^{65}$ and splicing regulatory proteins. Therefore, PTB binding might antagonize, at least in some instances, splice site recognition by $U2AF^{65}$ (Sauliere et al., 2006). In addition to that, PTB is known to be involved in other aspects of mRNA metabolism such as mRNA localization and stability, 3' end processing, and internal ribosome entry site (IRES)-mediated translation (Sawicka et al., 2008).

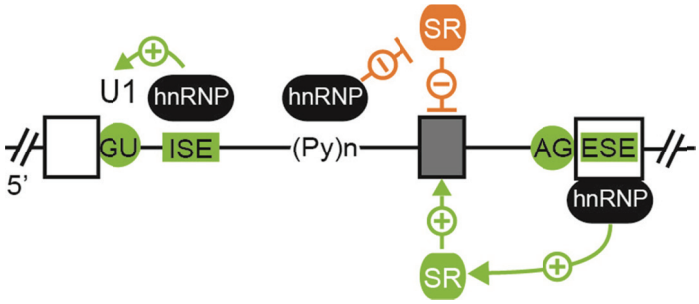

Figure 2: Exemplary scheme of combinatorial action of hnRNPs and SR proteins. Cassette exon (gray) inclusion is mediated by the recruitment of spliceosomal components or positive (green) factors through hnRNPs, or by displacement of a negatively-acting (orange) SR protein. A detailed description of the combinatorial action of splicing regulators can be found in the review by Wachter et al. (2012). ESE, exonic splicing enhancer; ISE, intronic splicing enhancer. Scheme modified from Wachter et al. (2012).

The human genome encodes three PTB homologues, of which the two PTB-related genes, neuronal PTB (nPTB) and regulator of differentiation 1

(ROD1), display 70 - 80% homology to PTB. In humans, PTB and nPTB are involved in the concerted and coordinated regulation of neuronal development by triggering a switch in the neuronal AS program, highlighting the biological significance of PTBs as splicing regulators (Boutz et al., 2007b). In addition, *PTB* expression is regulated by a negative feedback control where binding of PTB to its own pre-mRNA results in exon skipping which introduces a PTC and subsequent mRNA turnover via NMD (Wollerton et al., 2004). It is well established that other alternatively spliced, nonproductive NMD targets are generated by auto-regulatory circuits of RNA binding proteins influencing splicing of their own pre-mRNAs to inhibit inappropriately high levels of protein production (Jumaa and Nielsen, 1997; Sureau et al., 2001; Stoilov et al., 2004; Cuccurese et al., 2005; Ni et al., 2007).

To induce neuronal development, PTB and nPTB are expressed in a mutually exclusive manner and as long as PTB protein levels are high, *nPTB* expression is repressed by the already described splicing-dependent PTB-mediated inhibition. Cell type-specific PTB expression is achieved by a tissue-specific microRNA directed against PTB (Makeyev et al., 2007), allowing nPTB expression in differentiated neurons, muscle, and testis. PTB itself is expressed in neuronal progenitor cells and glia cells (Boutz et al., 2007a; Boutz et al., 2007b; Spellman et al., 2007). The third homologue ROD1 is restricted to hematopoietic cells and PTB and nPTB are promoting the non-productive splicing of *ROD1* (Spellman et al., 2007).

In plants, PTB orthologues can be found as well, although they were poorly investigated at the beginning of this thesis work and their molecular function remained unclear. Few studies on plant PTBs were done and a previous work using pumpkin as model organism could demonstrate that the pumpkin PTB protein RBP50 is part of a phloem mobile RNP complex (Ham et al., 2009). It was shown that RBP50 might play a role in the delivery of specific transcripts to distantly located tissues; and all transcripts found in the RBP50-RNP complexes contained PTB-binding motifs. Thus, RBP50 was suggested to function as a core protein in the formation of specific RNP complexes (Ham et al., 2009), which is furthermore dependent on the

phosphorylation status of RBP50 (Li et al., 2011). The work by Wang and Okamoto (2009) addressed the function of PTBs in the model organism *Arabidopsis thaliana*. In their study, two PTB homologues were analyzed and based on expression studies and mutant analysis, the authors proposed that the respective proteins are involved in pollen germination. The simultaneous knockout of both genes was shown to be lethal and promoter studies indicated that the two PTBs are highly active in vegetative tissues and in mature pollen grains (Wang and Okamoto, 2009). The authors hypothesized that PTBs might be involved in RNA processing and translational control of stored mRNAs to supply the germinating pollen with proteins. Alternatively, it was discussed that PTBs could mediate the intracellular transport of mRNAs to provide the basis for cell polarity necessary for single tube extrusion (Wang and Okamoto, 2009). However, the molecular function and the putative splicing regulatory role of Arabidopsis PTBs remained unclear at the beginning of my thesis work.

The following review article provides a general overview of the role of polypyrimidine-tract binding proteins and other hnRNPs in alternative splicing regulation in plants:

A. Wachter, C. Rühl, **E. Stauffer** (**2012).** *The role of polypyrimidine-tract binding proteins and other hnRNP proteins in plant splicing regulation.* Frontiers in Plant Science 3:81.

1.4. Aims of the work

The global analysis of pre-mRNA splicing in plants revealed that the occurrence of AS is widespread, however, the detailed underlying control mechanisms by splicing regulatory proteins, and the functional impact of alternative transcript variants on gene expression, remained unclear. At the beginning of my work, only few reports about plant PTBs were available and it was not investigated if plant PTBs have a splicing regulatory function to regulate a whole set of target transcripts as described for their orthologues in humans. The main objective of this work was to analyze the role of plant PTBs in AS and to investigate if AtPTBs have the potential to regulate their own expression in an auto- and cross-regulatory circuit. Therefore, we started with the detailed analysis of the potential auto- and cross-regulation of plant PTB expression in the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana*. Having established that AtPTBs regulate their own expression by a switch in pre-mRNA splicing, we were able to link AS with the mRNA surveillance mechanism nonsense-mediated decay (NMD) and we could demonstrate that one alternative transcript variant of each AtPTB is targeted by NMD. Furthermore, polypyrimidine stretches are often found between the branch point and 3' splice sites so that PTB-dependent regulation might interfere, at least in some instances, with binding of the splicing auxiliary factor $U2AF^{65}$. Thus, we were interested to test if AtPTB binding antagonizes $U2AF^{65}$ pointing towards the mechanistic aspect of AtPTB action in splicing regulation. In addition to their role in splicing regulation, PTBs are known to be involved in various cellular processes unrelated to splicing. Therefore, we investigated their subcellular localization pattern and we hypothesized that AtPTBs might have a function in a translational control mechanism as the proteins were found to localize to different cellular compartments and can regulate gene expression in a splicing-independent manner.

The second objective of this work was to analyze if AtPTBs are regulators of a global splicing program and by the use of AtPTB misexpression lines we were able to investigate the splicing regulatory impact of AtPTBs in a

transcriptome-wide scale. Upon the identification of AtPTB-dependent AS events, we were able to link some of the PTB-regulated events to biological functions. In this project, my main objective was the generation of polyclonal antibodies specifically detecting the individual AtPTB proteins and to monitor their levels in the corresponding AtPTB misexpression lines.

2. Results

2.1. Arabidopsis PTBs underlie regulatory circuits based on alternative splicing and downstream control

2.1.1. The Arabidopsis genome encodes three PTB-like genes

The Arabidopsis genome was analyzed for the presence of proteins orthologous to human PTB (Genbank accession number NM_002819) using BLAST (Altschul et al., 1997) and three PTB-like genes were identified. The genes *At1g43190*, *At3g01150*, and *At5g53180* share 28 – 34% identical and 43 – 51% similar amino acids (Table 1) with human PTB and all three Arabidopsis PTB orthologues (AtPTBs) seem to have at least three RRMs. Interestingly, AtPTB1 (*At3g01150*) and AtPTB2 (*At5g53180*) are close homologues sharing 74% identical and 86% similar amino acids. In contrast to that, AtPTB3 (*At1g43190*) is less related to them having only 30 – 32% identical and 53% similar amino acids, respectively.

Table 1: Amino acid sequence homology of AtPTBs and HsPTB. Numbers indicate percentage of identical and similar amino acids, respectively.

	AtPTB1	AtPTB2	AtPTB3	HsPTB
AtPTB1	100%			
AtPTB2	74%, 86%	100%		
AtPTB3	32%, 53%	30%, 53%	100%	33%, 51%
HsPTB	34%, 50%	28%, 43%	33%, 51%	100%

As shown in the gene model (Figure 3), AS of *AtPTB* pre-mRNAs results in two distinct splicing variants. In the case of *AtPTB1* and *AtPTB2* pre-mRNA splicing, skipping of the cassette exon generates splicing variant I (SPI) representing the protein coding isoform, whereas retention of the cassette exon results in splicing variant II (SPII). Importantly, SPII transcript variants harbor a PTC targeting those transcript isoforms for degradation by NMD.

For *AtPTB3* pre-mRNAs, the retention of the cassette exon leads to the protein coding transcript variant, whereas cassette exon skipping introduces a PTC reflecting the situation found for mammalian PTB. The occurrence of the two splicing variants for each *AtPTB* gene was experimentally validated in Arabidopsis wild type (WT) plants and an NMD-deficient mutant, which showed an over-accumulation of the SPII transcript variants proving that AS of *AtPTBs* is linked to NMD.

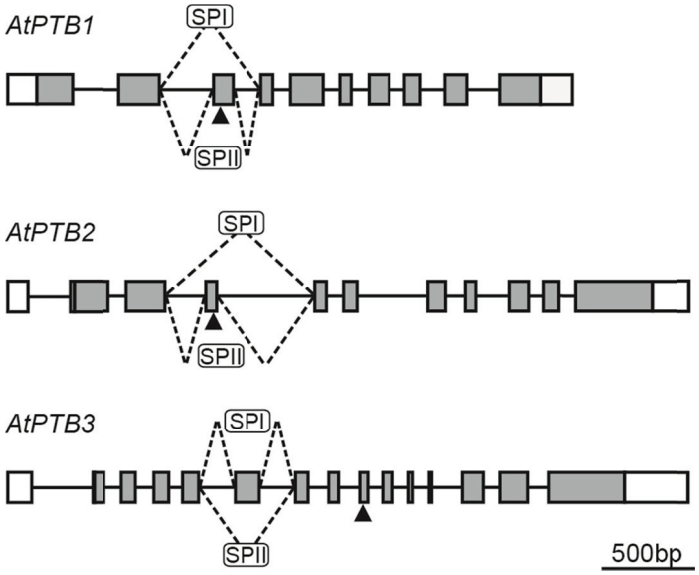

Figure 3: Gene model of Arabidopsis PTBs. Exon-intron structure of the three PTB-like genes. Exons are represented by boxes and introns by lines. The dashed lines indicate the AS events leading to SPI and SPII transcript variants. The coding sequence of SPI transcripts is shaded in grey whereas the triangles indicate the site of the premature termination codons in SPII transcript variants. Scheme modified from Stauffer et al. (2010).

2.1.2. *AtPTB* expression is regulated by auto- and cross-regulation

The expression of mammalian *PTB* is regulated by AS and it is well documented that PTB binds to its own mRNA leading to cassette exon skipping and introduction of a PTC (Boutz et al., 2007b; Wollerton et al., 2004). Furthermore, PTB has been reported to suppress expression of *nPTB* by a comparable AS-dependent mechanism (Boutz et al., 2007b). Therefore,

we were interested if AtPTBs show a similar regulation and thus have an AS-dependent regulatory capacity in plants as well.

To test this hypothesis, we fused the genomic 5' region of the three *AtPTB* genes, including the alternatively spliced part, in frame to enhanced green fluorescent protein (EGFP). This reporter construct allowed splicing to the SPI transcript variant, resulting in strong EGFP fluorescence, and to SPII transcripts that should be turned over by NMD. Afterwards, the reporter constructs were transiently expressed in *Nicotiana benthamiana* leaves and total EGFP fluorescence was measured after co-expression of *PTB* cDNA sequences. In conclusion, we were able to demonstrate that AtPTB proteins regulate their own expression in an AS-dependent manner. Furthermore, the two close homologues, AtPTB1 and AtPTB2, had the ability to cross-regulate reporter activity in our transient system via a change in AS. In contrast to that, AtPTB3 auto-regulated its own expression via a change in the splicing of its own mRNA, whereas it did not influence splicing of the *AtPTB2*-based reporter construct. Interestingly, we identified a splicing-independent repression of reporter activity by AtPTB proteins indicating the existence of other regulatory functions of plant PTBs, which might, for example, involve translational repression. To have a closer look at the splicing-independent regulatory function of Arabidopsis PTBs, the alternatively spliced region based on the SPI cDNA sequence of the three *AtPTBs* was fused in frame to EGFP and reporter activity was analyzed as described before. The results obtained with the fully spliced reporter further supported the assumption that AtPTBs can also act downstream of splicing control.

The so far described data had been generated using a transient system with massive overexpression of the reporter constructs and proteins, and we wanted to expand our knowledge to Arabidopsis to investigate the AS-dependent regulation of AtPTBs. In detail, we expressed the coding sequence (cds) of *AtPTB2* under control of the cauliflower mosaic virus (CaMV) promoter to allow a robust overproduction and accumulation of AtPTB2 protein. In contrast to that, an artificial micro RNA (amiRNA) approach has been used to reduce total *AtPTB2* transcript and protein levels. Subsequently, the relative ratios of SPII to total *AtPTB2* transcripts from the

endogenous gene were determined in WT and stably transformed Arabidopsis plants. In line with our hypothesis, the down-regulation of total *AtPTB2* transcript levels resulted in a clear reduction of the relative SPII transcripts, whereas upon overexpression of AtPTB2 protein, elevated SPII transcript levels were observed. We also extended this analysis to AtPTB1 and determined the relative transcript ratio of *AtPTB1* SPII to total transcript levels. Interestingly, we observed a similar effect for *AtPTB1* derived transcripts which was less pronounced compared to *AtPTB2* transcript levels, but further supported the cross-regulation of AtPTB1 and AtPTB2 expression. By the generation of AtPTB-specific polyclonal antisera, we could verify the interlocked feedback regulation on protein level as well (Rühl et al., 2012) and further details can be found in the chapter 2.2.1. In conclusion, we demonstrated that PTBs from Arabidopsis underlie an AS-dependent auto- and cross-regulation, which is directly reflected by total *AtPTB* expression and relative SPII transcript levels.

2.1.3. U2AF^{65} antagonizes AtPTB-mediated splicing regulation

To study the mechanistic aspects of AtPTB-mediated splicing decisions, we tested if the splicing auxiliary factor U2AF^{65} interferes with AtPTB2- and AtPTB3-mediated AS control of the reporter constructs. U2AF^{65} is known to bind to polypyrimidine-rich stretches that are typically found between the intronic branch point and the 3' splice site. Indeed, we observed a reduced formation of SPII mRNAs after co-expression of U2AF^{65} and thus an increase in reporter fluorescence. Hence, an antagonistic effect of these splicing factors could be demonstrated indicating that U2AF^{65} and AtPTB2/AtPTB3 either compete for binding to certain polypyrimidine tracts or the proteins affect their regulatory properties through protein-protein interaction.

In addition to the competition of U2AF^{65} with AtPTBs in controlling splicing decisions, we tested whether human PTB can alter splicing of the reporter constructs and thus is able to recognize plant derived polypyrimidine stretches and to interfere with the plant splicing machinery. Interestingly, we were able to show that co-expression of human PTB completely altered

splicing of the AtPTB-based reporter constructs and that the resulting splicing products corresponded mainly to intron retained AS variants. Furthermore, we observed a toxic effect of human PTB, which resulted in leaf lesions upon transient expression, pointing towards a massive interference with the plant splicing machinery. Thus, PTBs from different clades have conserved as well as variable target sequences and PTBs from different organisms share common regulatory features.

2.1.4. AtPTBs localize to diverse cellular compartments

Our results indicated that AtPTBs are involved in different processes that are splicing-dependent as well as splicing-independent. As the nucleus is the site where splicing occurs and PTBs are splicing regulatory proteins, we expected them to be localized in the nuclear compartment. However, based on our analyses of the splicing-independent reporter constructs, we hypothesized that AtPTBs might be involved in a translational repression mechanism that would be expected to take place in the cytosol. Therefore, we generated fluorescent fusion proteins and performed *in vivo* localization studies upon transient expression in Arabidopsis protoplasts. All three AtPTB-EGFP fusion proteins could be detected in the nucleus and cytosol, which was confirmed by the co-localization with nuclear and cytosolic localized DsRED, respectively. Interestingly, in a significant proportion of protoplasts, AtPTB1- and AtPTB2-EGFP were found in cytoplasmic foci, in addition to their localization to the cytoplasm and nucleus. These dot-shaped structures have been identified as processing bodies (P-bodies) using a reporter fusion with the well-known P-body marker decapping enzyme 1 (DCP1) (Xu et al., 2006). In addition to that, the total number of cells displaying P-body localization of AtPTB1-YFP and AtPTB2-YFP increased upon co-expression of DCP1-CFP. In contrast to AtPTB1- and AtPTB2-YFP, AtPTB3-YFP localized to the nucleus and cytosol, and only upon co-transformation of DCP1-CFP the protein was detected in P-bodies as well. This change in the localization of AtPTB3-YFP might be explained by the fact that P-bodies are described as highly dynamic, variable structures, and that

P-body formation depends on its constituents. Due to the massive overexpression of DCP1-CFP the total number and size of P-bodies increased and this might trap more AtPTB proteins in these structures.
Subsequently, we extended our analysis to Arabidopsis seedlings, which stably expressed the AtPTB-EGFP fusions under the CaMV promoter. In line with the localization patterns of the AtPTB-EGFP proteins in the protoplast system, the AtPTB fusions were found in the cytoplasm and nucleus of epidermal and guard cells. In addition, AtPTB1- and AtPTB2-EGFP were observed in dot-shaped structures in the cytoplasm that probably represent P-bodies.

All results addressing the auto- and cross-regulation and localization of AtPTBs are described in detail in the following publication:

E. Stauffer , A. Westermann, G. Wagner, A. Wachter **(2010).** *Polypyrimidine tract-binding protein homologues from Arabidopsis underlie regulatory circuits based on alternative splicing and downstream control.* The Plant Journal 64:243–255.

2.2. AtPTBs from Arabidopsis are key regulators of alternative splicing with implications in fundamental processes

2.2.1. Generation and analyses of AtPTB misexpression lines

To identify potential targets of AtPTB-mediated splicing regulation, transgenic Arabidopsis lines with altered AtPTB protein levels were generated. For overexpression of AtPTBs, constructs bearing the cds of the individual *AtPTB* genes under control of the constitutive CaMV promoter were transformed into Arabidopsis Columbia-0 WT plants, and transgenic lines with maximum *AtPTB* transcript levels were selected for further analyses. To down-regulate *AtPTB* expression an amiRNA approach was chosen. We used amiRNAs that were directed against either the individual *AtPTBs* or the two closely related homologues *AtPTB1* and *AtPTB2* simultaneously (ami1&2). After detailed analyses of the amiRNA lines with respect to total *AtPTB* transcript levels, transgenic plants with minimum *AtPTB* transcript levels, showing a reduction to 30 - 40% in comparison to WT, were selected for our further experiments. In addition to the quantification of total *AtPTB* transcript levels, we wanted to observe protein accumulation and reduction in the respective misexpression lines. Therefore, we generated polyclonal antisera directed against each AtPTB, and after affinity purification of the raw sera, we were able to show their specificity using extracts from *N. benthamiana* plants transiently expressing the individual AtPTBs. For AtPTB2- and AtPTB3-specific antibodies, we could demonstrate that the purified antibodies were highly specific for the individual AtPTB proteins and strong immune signals were detected. In contrast to that, the antibody directed against AtPTB1 resulted in a cross-detection of the closely related AtPTB2 protein and the immune signal was overall weaker. Detailed analyses of the AtPTB misexpression lines in Arabidopsis seedling as well as rosette leave samples revealed that downregulation of AtPTB1, AtPTB2, and AtPTB3 resulted in a complete loss of the immune signal whereas the AtPTB overexpressing plants accumulated the proteins to a great extent (except for AtPTB3 where only a 1.5-fold increase on protein level was observed). Importantly, the already described cross-regulation between AtPTB1 and AtPTB2 was nicely

verified on protein level using the AtPTB misexpression lines. For example, the massive overexpression of AtPTB1 resulted in a nearly complete downregulation of AtPTB2 protein. Furthermore, the extent of the auto- and cross-regulation between the individual AtPTB proteins varies during development: While no pronounced change of AtPTB3 protein was detected upon AtPTB1 and AtPTB2 misexpression in seedling stage, the over-expression of AtPTB2 in rosette leaves reduced the level of AtPTB3 protein and a reciprocal change was detected in the ami1&2 line. In summary, the detailed analyses of the AtPTB misexpression lines revealed that we altered AtPTB protein levels markedly, making these lines suitable for our downstream transcriptome-wide splicing studies. In addition, future studies addressing the developmental aspects of AtPTB-dependent auto- and cross-regulation will help to further investigate specific as well as redundant functions of the AtPTB proteins.

2.2.2. Transcriptome-wide splicing studies reveal a widespread AS-regulatory role of AtPTBs

With the help of RNA-seq and stringent bioinformatical analyses we were able to identify a set of transcripts showing changes in their AS due to the misexpression of AtPTBs. In detail, we filtered for AS events with preferential reciprocal changes in the amiRNA and overexpressing lines and subsequently filtered for changes between WT and amiRNA samples. This computational pipeline helped us to exclude AS events that only changed upon AtPTB overexpression and thus should reduce the number of events that changed due to the excessive overproduction of a splicing factor. Thereby, we were able to identify 452 AS events that were regulated by the close homologues AtPTB1 and AtPTB2. Among those 452 AS events, we found an overrepresentation of cassette exons and alt. 5’ events compared to all AS events. In contrast, the relative fraction of alt. 3’ events was decreased seven fold. For experimental validation of the AtPTB-regulated events, target transcripts of the different AS type categories were selected and we were able to verify the authenticity of the computationally predicted

AtPTB regulation candidates in every case tested. Applying the same computational pipeline as for the AtPTB1 and AtPTB2 misexpression lines, we could identify only five AtPTB3-dependent events and only two of those could be experimentally verified. Therefore, we conclude that AtPTB1 and AtPTB2, but not AtPTB3, have a widespread function in the regulation of AS in Arabidopsis.

2.2.3. AtPTB1- and AtPTB2-regulated AS events have functional implications in diverse biological processes

Among the candidate genes that were regulated in an AtPTB-dependent manner, was the previously investigated phytochrome interacting factor 6 (PIF6) which was shown to generate two transcript variants upon AS where both isoform most likely result in different protein versions (Penfield et al., 2010). The *PIF6* pre-mRNA sequence harbors a cassette exon and Penfield et al. (2010) could show that constitutive overexpression of the exon skipped splicing variant, but not the exon included isoform, resulted in primary seed dormancy. Thus, *PIF6* splicing was analyzed in various WT tissues e.g. rosette leaves, buds, and flowers, and we could find a tissue-specific change in *PIF6* AS. We also analyzed *PIF6* splicing in the AtPTB misexpression lines and we could show that AtPTB misexpression perturbed the tissue-specific AS of *PIF6* completely. In addition to that, changes in *PIF6* AS coincided with altered rates of abscisic acid (ABA)-dependent seed germination. For example, in presence of exogenous ABA the simultaneous down-regulation of *AtPTB1&2* resulted in an elevated germination rate. In contrast to that, the reciprocal effect was found upon overexpression of AtPTB1, resulting in a delayed germination behavior. To proof the direct binding of AtPTB2 to the *PIF6* target mRNA we performed an electrophoretic mobility shift assay (EMSA). The alternatively spliced region of *PIF6* was used as probe, and a direct binding of recombinantly expressed AtPTB2 protein to the *PIF6* RNA probe could be shown.

The list of AtPTB1/2 regulation targets also included the two flowering regulatory genes flowering locus K (FLK) and flowering locus M (FLM). FLK

is known to positively regulate flowering as a repressor of flowering locus C (FLC) (Lim et al., 2004; Mockler et al., 2004) and a link between AS of *FLK* and expression of FLC was reported previously (Deng et al., 2010). The knockdown of *AtPTB1&2* caused an increase in the *FLK* intron retention variant whereas overexpression of AtPTB1 had the reciprocal effect. Thus, one would expect an increased *FLC* transcript level upon *AtPTB* downregulation. However, we found the opposite change, with *FLC* transcript levels being below the detection limit upon *AtPTB1&2* downregulation. Furthermore, AS of *FLM* was shown to result in two splicing variants one of which was previously shown to encode a floral repressor (Ratcliffe et al., 2001) and an enrichment of this transcript variant was observed in the *AtPTB1/2* knockdown lines. Taken together, due to the changes in AS patterns of central flowering-regulating factors, we expected a flowering phenotype in the *AtPTB1&2* knockdown plants. However, the onset of flowering was unchanged compared to WT plants pointing towards additional control mechanisms or a compensation of changes in the levels of positive and negative regulators of flowering.

All results addressing the transcriptome-wide regulation of AtPTBs and their functional implications in the different developmental processes can be found in the following publication:

C. Rühl, **E. Stauffer**, A. Kahles, G. Wagner, G. Drechsel, G. Rätsch, A. Wachter (**2012).** *Polypyrimidine tract-binding protein homologues from Arabidopsis are keyregulators of alternative splicing with implications in fundamental developmental processes.* Accepted in Plant Cell.

3. Discussion

3.1. AtPTB expression is regulated by the coupling of AS and NMD

Our work addressing the auto- and cross-regulation of AtPTBs showed that all three Arabidopsis *PTB* genes generate two types of splicing variants, one leading to full-length protein production, whereas the other transcript variant is subjected to NMD. Furthermore, our work established that Arabidopsis PTBs have a splicing-regulatory function that can alter AS of their own pre-mRNAs and we proposed a model of negative auto- and cross-regulation, as elevated AtPTB protein levels changed AS in favor of the PTC-containing transcript variant (Figure 4). Thus, PTBs from mammals (Wollerton et al., 2004) and plants (Stauffer et al., 2010) are subject to auto- and cross-regulatory circuits involving AS coupled to NMD for fine-tuning their expression. In line with the findings of *PTB* pre-mRNA splicing in Arabidopsis, the work by Wang and Brendel (2006) suggested the existence of equivalent *PTB* splice variants in *Oryza sativa,* pointing towards an evolutionary conservation of *PTB* AS in plants.

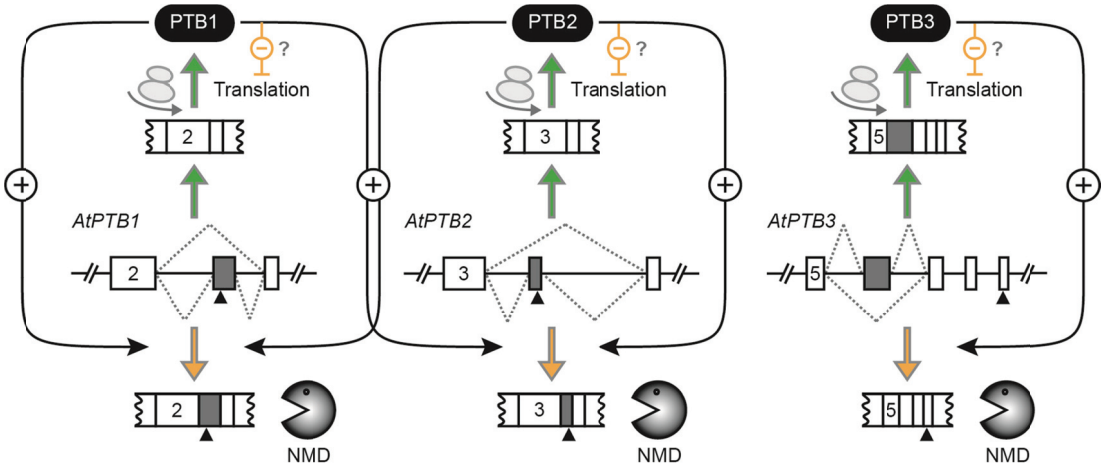

Figure 4: Auto- and cross-regulation of Arabidopsis PTB homologues. Displayed are the alternative splicing events (dotted line) for the *AtPTB1*, *AtPTB2*, and *AtPTB3* pre-mRNAs. Exons are depicted as boxes, introns as lines, gray boxes

Figure legend continued: refer to cassette exons, which are either skipped (AtPTB3) or included (AtPTB1/2), resulting in the introduction of a premature termination codon (black triangle) and targeting those transcripts for degradation by nonsense-mediated decay (NMD). The splicing variants shown on top of each gene model are translated into the PTB proteins, which trigger splicing of pre-mRNAs to the NMD target variants in an auto- and cross-regulatory manner. Modified from Wachter *et al.* (2012).

Interestingly, a similar gene expression control mechanism has been revealed for other RNA binding proteins like the GRPs and SR proteins, indicating that AS-coupled NMD plays a more global role in gene expression control. For example, AtGRP7 and AtGRP8 were shown to underlie an interlocked feedback loop that is linked to the unproductive splicing of their pre-mRNAs (Schoning et al., 2008) and the work by Palusa and Reddy (2010) revealed that coupling of AS and NMD is extensively used to control SR gene expression. The authors could demonstrate that out of 53 PTC-containing SR transcripts, about one half was turned over by NMD.

New techniques together with increasing sequence support helped to elucidate the global impact of AS-coupled NMD. A high-resolution reverse transcription PCR panel (RT-PCR) was used to analyze AS of 270 selected genes and of those, 32% turned out to generate NMD targets (Kalyna et al., 2012). Interestingly, a transcriptome-wide study by Drechsel et al. (G. Drechsel, A. Kahles, A.K. Kesarwani, E. Stauffer, J. Behr, P. Drewe, G. Rätsch, A. Wachter, unpublished data) could show that AS-coupled NMD occurs in 17.5% of all multi-exon, protein-coding genes in Arabidopsis and this number still represent an underestimation as only unstressed seedling samples were tested. Taken together, all aforementioned publications provide evidence that the coupling of AS and NMD is used to control expression of various genes including splicing factors and helps to fine-tune the plant transcriptome.

3.2. Alternative splicing control by AtPTBs

The transcriptome-wide study of AtPTB-dependent AS in Arabidopsis revealed a global role of AtPTBs in splicing regulation. In line with the number of PTB-regulated AS events described by Llorian et al. (2010) in human, we were able to identify a similar number of regulation targets, comprising 452 AS events derived from 308 distinct genes (Rühl et al., 2012). Among those, we observed a clear over-representation of alt. 5' events and cassette exons. Downregulation of AtPTB1&2 resulted in skipping of most cassette exons, thus revealing that the inclusion of those is AtPTB1/2-dependent. Our previous analyses addressing the auto- and cross-regulation of AtPTBs (Stauffer et al., 2010), showed a comparable underlying AtPTB-dependent splicing regulation: Elevated AtPTB1/2 protein levels changed AS in favor of the PTC-containing transcript variant and retention of these poison exons targeted them for degradation. Given that a significant fraction of the AtPTB-regulated AS events resulted in transcripts with NMD-eliciting features, a more widespread role of AtPTB-dependent AS regulation and mRNA turnover can be anticipated. In addition to its role in cassette exon regulation, human PTB was shown to be involved in the repression of intron splicing of neuron-specific genes (Yap et al., 2012). Thereby, PTB achieves nuclear retention and degradation of the regulated transcripts and thus coordinates the expression of functionally linked genes (Yap et al., 2012). Accordingly, AtPTB1/2 were found to promote intron splicing as well as intron retention and the splicing regulators impacted on the choice of 5' splice sites whereas the selection of 3' splice sites was underrepresented among the AtPTB1/2-regulated AS events. Taken together, we observed a preferential regulation of certain AS events by AtPTB1&2 and by the analysis of single amiRNA lines, we were able to show that the two homologues have specific and redundant splicing regulatory functions. Interestingly, based on our transcriptome-wide analysis, we concluded that AtPTB3 does not play a major role in the regulation of pre-mRNA splicing, and rather might be involved in other mRNA-associated processes like transport of mRNA particles. This hypothesis is supported by a work from Ham et al. (2009), in which the pumpkin PTB3 homologue RBP50 was shown to be part of a

phloem-mobile RNP complex. Furthermore, phosphorylation of RBP50 is required for the assembly of the phloem-mobile RNP complexes (Li et al., 2011). Future work needs to investigate if AtPTB3 is involved in long distance transport of mRNAs and if the protein also has to be modified post-translationally to fulfill its function. Still the question why plants possess three PTB proteins, of which two are close homologues with specific and redundant implications in splicing control, remains to be elucidated. One could speculate that the expression of AtPTB1 and AtPTB2 might alter during development or in various cell types so that plants can respond to environmental cues specifically. Some pilot experiments using the AtPTB-specific antibodies already indicated that protein levels change in different plant tissues and further experiments will help to understand the specific roles of Arabidopsis PTBs in more detail. In addition, post-translational protein modifications like phosphorylation or methylation might be necessary for protein function and thus could help to adjust PTB activity.

3.3. AtPTBs function as activator and repressor of AS events

In our transcriptome-wide sequencing data, AtPTB-repressed and AtPTB-activated AS events were identified. For a long time PTB was considered as a general splicing repressor as PTB binding most often resulted in skipping of cassette exons (Spellman and Smith, 2006). However, it is now becoming evident that the mode of PTB action usually depends on the binding position within the pre-mRNA (Xue et al., 2009; Llorian et al., 2010). Two recent publications addressed the binding position-dependent splicing action of PTB and new conclusions on PTB action were put forward. Xue et al. (2009) used cross-linking immunoprecipitation coupled with high-throughput RNA sequencing (CLIP-seq) to identify PTB binding positions in a transcriptome-wide manner. The authors could show that PTB binding close to alternative splice sites generally induced skipping of the respective exons and inclusion was achieved if PTB was binding in the proximity of constitutive splice sites (Xue et al., 2009). This work is in line with previous results regarding PTB as a general splicing repressor and inclusion of alternative exons occurs only

upon weakening of the constitutive splice site. In contrast to that, Llorian et al. (2010) could show that PTB indeed also functions as a direct splicing activator. *PTB* knockdown lines were analyzed using high-density oligonucleotide splice-sensitive microarrays and they demonstrated that the enrichment of distinct sequence motifs around the regulated AS event directly reflects if the exon is PTB-activated or PTB-repressed. In the case of PTB-repressed exons, that still represent the major proportion of PTB-dependent splicing events in human, polypyrimidine-rich sequences were found upstream or within the cassette exon. In contrast to that, PTB-activated exons had PTB binding sites downstream of the cassette exons, pointing towards a direct role of PTB in the activation of exon inclusion rather than a weakening of constitutive splice sites (Llorian et al., 2010). The discrepancies between the two publications might be ascribed to the different methods used to identify PTB-regulated AS events. Therefore, the datasets were combined and the motif enrichment described by Llorian et al. (2010) could be resembled for the AS events described by Xue et al. (2009). In conclusion, PTB seems to be able to activate cassette exon inclusion in at least two ways: on one hand by the weakening of constitutive splice sites (Xue et al., 2009), and on the other hand, the direct activation of cassette exon inclusion by PTB binding downstream of the regulated cassette exon (Llorian et al., 2010). Future work needs to address the detailed binding mode of plant PTBs to their target mRNAs helping to elucidate if PTB-activated and PTB-repressed AS events are also reflected by the relative PTB binding position to the regulated AS event.

3.4. Splicing-independent functions and subcellular localization of AtPTBs

Pre-mRNA splicing is confined to the nuclear compartment but it has been shown previously that hnRNPs are subject to nucleo-cytoplasmic shuttling, which can be regulated by post-translational protein modifications. For example, the nucleo-cytoplasmic transport of PTB is achieved by the 3',5'-cAMP-dependent protein kinase (PKA) and phosphorylation of PTB is a prerequisite for its export to the cytosol (Xie et al., 2003). In addition to phosphorylation, it is reported that methylation of arginine residues of hnRNPs modulates their nuclear localization (Chang et al., 2011). In line with the nucleo-cytoplasmic shuttling of hnRNPs, our fluorescent fusion protein studies of AtPTBs established the localization of Arabidopsis PTBs to diverse cellular compartments. In addition to the nuclear and cytosolic localization, AtPTB1 and AtPTB2 were found to localize to P-bodies. In general, P-bodies are membrane-free structures, where mRNAs can be turned over as suggested by the enrichment of decapping enzymes, activators of decapping, and exonucleases (Parker and Sheth, 2007). Previous studies on P-body behaviour in yeast showed that P-body-localized mRNAs can either be degraded or re-enter the polysomal fraction, such that P-bodies are also used as sites to store mRNAs for a temporary translational arrest (Brengues et al., 2005; Parker and Sheth, 2007). One could speculate that AtPTBs are not only involved in the regulation of AS in a transcriptome-wide manner, but rather have multiple functions in the control of gene expression. Future work needs to address the mechanism of translational control in more detail. Here, the question is whether AtPTBs are involved in an active retraction of mRNAs from the polysomal pool or if the P-body localization is dependent on other cellular cues like defects in translation initiation or termination (Parker and Sheth, 2007). Moreover, as AtPTBs bind sequence-specific to their target mRNAs, they could represent important regulators to recruit the general repression/decay machinery and thereby modulate mRNA fate. Furthermore, it would be interesting to see whether specific post-translational modifications can be linked to the subcellular localization of Arabidopsis

PTBs helping to increase the repertoire of plants to fine-tune AtPTB-dependent outputs to internal and external cues.

3.5. Biological implications of AtPTB-dependent alternative splicing

Transcriptome-wide analyses of AtPTB-dependent splicing regulation resulted in the identification of many candidate genes with diverse functions in fundamental biological processes. So far, we were able to elucidate the consequences of AtPTB-mediated changes in AS in two selected biological processes. A previous publication showed that AS of *PIF6* pre-mRNAs resulted in two transcript variants of which one isoform lacked the DNA-binding domain. The overexpression of this shorter protein variant reduced primary seed dormancy (Penfield et al., 2010). We observed that upon *AtPTB* misexpression, AS of *PIF6* was significantly changed and a tissue-specific splicing switch was disrupted. Intriguingly, Arabidopsis lines with altered AtPTB protein levels had a germination phenotype in the presence of exogenous ABA. Previous studies already reported a link between AS and ABA signaling and for example, SR proteins were shown to be involved in the regulation of ABA signaling (Duque, 2011). In addition, a key player of seed maturation is abscisic acid insensitive 3 (*ABI3*) and a developmentally regulated AS switch was reported at the end of seed maturation (Sugliani et al., 2010). Furthermore, proper *ABI3* splicing is regulated by the newly identified splicing factor suppressor of *abi3-5* (SUA) that has been shown to interact with U2AF^{65}, and SUA binding might interfere with early spliceosome assembly (Sugliani et al., 2010). Interestingly, the *sua* mutant germinated faster in the presence of exogenous ABA reflecting the situation of the *ami1&2* misexpression line. In conclusion, putative overlapping functions of AtPTBs and SUA remain speculative and future work needs to address the influence of AtPTBs on *ABI3* splicing and other aspects of ABA signaling.

Our transcriptome-wide splicing studies also revealed a role of AtPTBs in the expression control of flowering regulators. Plants need to integrate a whole set of environmental cues to control the time at which they flower. In the past

few years, it became evident that flowering time control is often associated with RNA processing-based regulatory mechanisms including alternative splicing (Terzi and Simpson, 2008; Deng et al., 2010). Interestingly, we showed that upon AtPTB misexpression, the splicing of the flowering regulators *FLK* and *FLM* changed significantly. For example, upon *AtPTB1&2* downregulation, AS of the floral activator *FLK* switched to a splice variant that should result in FLC induction. Unexpectedly, *FLC* expression was not even detectable upon the simultaneous knockdown of *AtPTB1* and *AtPTB2*. In addition, we could identify an altered splicing pattern of *FLM* pre-mRNAs with an increase of the splicing variant formerly described as a floral repressor (Ratcliffe et al., 2001). Although a flowering phenotype due to the massive changes in major regulators of flowering would be expected, the *AtPTB1&2* misexpression plants did not show an altered flowering behavior. Thus, we speculate that the changes in AS of floral activators and repressors somehow compensate each other or that further regulatory mechanisms become active. A possible scenario would be that further splicing regulatory proteins are involved in the regulation of the key regulators of flowering providing a platform to interconnect various environmental cues to ensure the tightly regulated onset of flowering.

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5 Publications

5.1. Research articles

5.1.1. **E. Stauffer**, A. Westermann, G. Wagner, A. Wachter, (**2010**) *Polypyrimidine tract-binding protein homologues from Arabidopsis underlie regulatory circuits based on alternative splicing and downstream control.* The Plant Journal 64:243–255.

Personal contributions

In the present work, I performed most of the cloning procedures, transient expression of constructs, and the subsequent analyses using fluorometer assays. In addition to that, I constructed all fluorescent fusion proteins and did the microscopic analyses. A. Westermann did initial work on AtPTBs and I had technical support from G. Wagner. Experimental design and writing of the manuscript was done together with A. Wachter.

5.1.2. C. Rühl, **E. Stauffer**, A. Kahles, G. Wagner, G. Drechsel, G. Rätsch, A. Wachter, **(2012)** *Polypyrimidine tract-binding protein homologues from Arabidopsis are key regulators of alternative splicing with implications in fundamental developmental processes.* Plant Cell, DOI 10.1105/tpc.112.103622

Personal contributions

In the present work, my contribution was the generation of the polyclonal antibodies. Therefore, AtPTB sequences were cloned in different expression vectors, recombinant proteins were expressed, purified, and sent for immunization to the company BioGenes in Berlin. After several immunization steps, the raw sera were tested for AtPTB-specific titers and subsequently, I affinity purified the raw sera against the individual AtPTBs. Subsequently, I performed the Western Blotting experiments for the characterization of the transgenic AtPTB misexpression lines and co-wrote the manuscript.

5.1.1. **E. Stauffer**, A. Westermann, G. Wagner, A. Wachter, (**2010)** *Polypyrimidine tract-binding protein homologues from Arabidopsis underlie regulatory circuits based on alternative splicing and downstream control.* The Plant Journal 64:243–255.

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Polypyrimidine tract-binding protein homologues from Arabidopsis underlie regulatory circuits based on alternative splicing and downstream control

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SUMMARY

Alternative splicing (AS) of precursor mRNAs is a widespread phenomenon in plants; however, many questions, especially regarding its regulation and functional implications, remain to be elucidated. In vertebrates, polypyrimidine tract-binding proteins (PTBs) have been identified as key splicing factors influencing splice site selection and orchestrating coordinated splicing programmes during developmental processes. Here, we analysed three *PTB* homologues from *Arabidopsis thaliana* and provide evidence for their gene regulatory potential based on AS and a splicing-independent mechanism. Our data reveal that Arabidopsis PTB homologues are subject to extensive auto- and cross-regulation via AS-coupled nonsense-mediated decay, thereby establishing a basis for interlinking their expression. Furthermore, the multiple modes of action of Arabidopsis PTB homologues are reflected in their subcellular localization in the nucleus, cytosol and processing bodies. This work provides insight into the regulation of AS in plants and highlights the regulatory potential of the multifunctional plant PTB homologues, which might have important implications in diverse biological processes.

Keywords: polypyrimidine, splicing, nonsense-mediated decay, P-body, gene regulation, Arabidopsis.

INTRODUCTION

Alternative splicing (AS) of precursor messenger RNAs (pre-mRNAs) is an intricately regulated step in the course of mRNA maturation, considerably contributing to the complexity of eukaryotic gene expression. In the co-transcriptional process of AS, the use of alternative splice sites within one pre-mRNA type results in the formation of transcript variants that retain different exon sequences and can exhibit distinct features. Based on the analysis of cDNA and expressed sequence tag (EST) data sets, AS has been estimated to occur for ~50% of human genes (Gupta *et al.*, 2004), and for ~20% of the genes in *Arabidopsis thaliana* and *Oryza sativa* (rice; Wang and Brendel, 2006). This discrepancy in AS proportions might be explained, at least to some extent, by much fewer available transcript data for plants compared with human (Reddy, 2007). Recent high-throughput RNA sequencing approaches suggest an even broader distribution of AS, with more than 90% of all human genes being affected (Wang *et al.*, 2008). Accordingly, deep sequencing of plant transcriptomes revealed AS rates of 42 and 33% for Arabidopsis (Filichkin *et al.*, 2010) and rice genes (Zhang *et al.*, 2010), respectively.

The functional implications of AS can mainly be observed in an increase in proteome diversity and an alteration of transcript properties, such as mRNA stability (Blencowe, 2006; Reddy, 2007). Using a comparative analysis, it was concluded that AS in plants has only a limited role in the functional expansion of the proteome (Severing *et al.*, 2009). However, approximately 40% of all AS events in plants result in aberrant transcript features such as a premature termination codon (PTC) (Wang and Brendel, 2006), which are expected to target those mRNAs for degradation by nonsense mediated decay (NMD) (Lejeune and Maquat, 2005). Coupling of AS and NMD turnover has been described as a means of gene regulation in mammals (Soergel *et al.*, 2006), and several recent studies indicate that similar mechanisms are active in plants. For example, as part of an auto-regulation

of the Arabidopsis RNA-binding protein AtGRP8, a PTC-containing splicing variant is generated and subjected to NMD (Schoning *et al.*, 2008).

Despite its wide distribution in higher eukaryotes, many fundamental questions about AS remain to be addressed. In particular, for the majority of AS events, neither the biological significance nor the underlying regulatory mechanisms have been established. However, now it is becoming evident that the complex control of AS is based on highly dynamic and flexible protein–RNA networks at different steps of spliceosome assembly (Matlin *et al.*, 2005; Chen and Manley, 2009). On one side this includes *cis*-regulatory elements in introns or exons that can enhance or silence the respective splicing events. On the other side, numerous regulatory factors have been described that interact with *cis* elements or protein components of the splicing machinery. Furthermore, RNA structures can directly control splicing decisions, e.g. by masking splice sites, as highlighted by the mechanisms of eukaryotic riboswitches (Wachter, 2010). Recently, another level of complexity has been uncovered by the finding that histone modifications can contribute to the regulation of AS (Luco *et al.*, 2010).

A widespread group of splicing regulators is the family of SR (serine-arginine) proteins that typically facilitate splice site recognition by binding to exonic splicing enhancers (Chen and Manley, 2009), but also display mRNA metabolic functions downstream of splicing (Zhong *et al.*, 2009). Interestingly, pre-mRNAs from SR proteins and other splicing regulatory factors themselves are subject to AS, thereby further expanding the repertoire of splicing factors (Reddy, 2007). Antagonistically to SR protein action, heterogeneous nuclear ribonucleoproteins (hnRNPs) commonly bind to splicing silencers, and thereby repress the use of splice sites (Chen and Manley, 2009).

In vertebrates, a well-studied member of the hnRNP family is the polypyrimidine tract-binding protein (PTB), which binds to CU-rich motifs in target pre-mRNAs, and thereby typically suppresses the inclusion of an adjacent exon (Sawicka *et al.*, 2008). Given its interaction with the polypyrimidine tract close to the 3′ splice site of target pre-mRNAs, PTB can interfere with the binding of splicing factors such as $U2AF^{65}$ in this region (Sauliere *et al.*, 2006). Alternative models of PTB-mediated exon suppression are based on its RNA looping activity, as well as on the inhibition of splice factor interactions required for exon or intron definition (Spellman and Smith, 2006). Mechanistic studies of different PTB-controlled splicing events suggested that these models are not mutually exclusive, but rather that the mode of PTB action depends on the AS event under control. A recent analysis of PTB–RNA interactions suggested a positional effect, with PTB binding close to a competing constitutive and an alternative site resulting in exon inclusion and skipping, respectively (Xue *et al.*, 2009). In addition to its splicing regulatory function, PTB is engaged in numerous other processes, such as polyadenylation, translation from internal ribosomal entry sites and transport, as well as stability of mRNAs (Sawicka *et al.*, 2008).

The regulatory potential and far-reaching functional implications of PTB action have been exemplified in humans, where a switch between PTB homologues coordinates a concerted AS programme during neuronal development (Boutz *et al.*, 2007b). These specific AS patterns are achieved by mutually exclusive expression of PTB and its neuronal paralogue nPTB, exhibiting distinct splicing regulatory functions. Furthermore, human PTB was shown to be auto-regulated in a negative feedback loop by AS of its own pre-mRNA (Wollerton *et al.*, 2004). In the presence of increased PTB levels, a higher proportion of *PTB* pre-mRNA is spliced to a PTC-containing NMD target transcript. Interestingly, a similar mechanism is found for auto- and PTB-mediated cross-regulation of nPTB in cell-specific splicing circuits during neuronal development (Boutz *et al.*, 2007b), as well as for repression of the third mammalian PTB homologue, ROD1, by PTB and nPTB (Spellman *et al.*, 2007).

For some plant introns, U-rich sequences have been shown to possess dual functions as polypyrimidine tract and UA-rich elements (Simpson *et al.*, 2004), both of which can be important in controlling splicing events (Simpson *et al.*, 2002). Whereas sequences of PTB-related proteins are found in plant genomes as well, only few reports are available on properties and functions of those plant homologues. Analysis of gene expression in egg cells and zygotes from maize indicated elevated transcript levels of a *PTB*-related gene upon *in vitro* fertilization (Okamoto *et al.*, 2005). In the recent work from Ham *et al.* (2009), a PTB homologue from pumpkin, RBP50, was found to be a major constituent of phloem-mobile ribonucleoprotein complexes. Interestingly, RBP50 is enriched in the phloem sap and can be translocated from source to sink tissues. Ham *et al.* (2009) isolated several RBP50-interacting phloem proteins as well as target mRNAs, and demonstrated that PTB specifically binds CU-rich motifs in these mRNAs. Furthermore, a role of two PTB-related proteins in Arabidopsis pollen germination was reported (Wang and Okamoto, 2009). Both genes were shown to be expressed in various vegetative tissues as well as in reproductive organs, including mature pollen, and for pollen grains a mutation in both genes led to reduced germination efficiency.

Despite these first reports on plant PTB homologues, many questions about their molecular functions, particularly as putative regulators of AS, as well as their biological role, remain to be addressed. In this work, we analyzed three PTB homologues from Arabidopsis and provide evidence for extensive auto- and cross-regulation of their expression. We show that PTB-mediated expression control is based on both AS and downstream, splicing-independent regulatory mechanisms. The multiple modes of action of Arabidopsis PTB homologues are further substantiated by their

subcellular localization in the nucleus, cytosol, and processing bodies (P-bodies). This work further highlights the versatile gene regulatory features of PTB proteins, and provides a basis for the elucidation of their biological functions in plants.

RESULTS

Alternative splicing of PTB homologues in Arabidopsis

The protein sequence of human PTB (Genbank accession number NM_002819) was used as a query in BLAST (Altschul *et al.*, 1997) to identify PTB orthologues from Arabidopsis. The three genes *AT1G43190*, *AT3G01150* and *AT5G53180* were found to encode PTB-related proteins displaying, compared with human PTB, 28–34 and 43–51% identical and similar amino acids, respectively (Table S1). A characteristic feature of mammalian PTBs is the presence of four RNA recognition motifs (RRMs) separated by three linker regions (Auweter and Allain, 2008). Using the Pfam database sequence search tool (Finn *et al.*, 2010), the presence of four RRMs in the protein derived from *AT1G43190* and three RRMs in proteins derived from *AT3G01150* and *AT5G53180* was predicted (data not shown), further supporting their identity as *bona fide* PTBs. The proteins encoded by *AT5G53180* and *AT3G01150* are close homologues exhibiting 74% identical and 86% similar amino acids, whereas the AT1G43190 protein shows a comparable level of conservation with the two other PTB-related proteins from Arabidopsis, as with human PTB (Table S1). In a previous report, *AT3G01150* and *AT5G53180* were designated *AtPTB1* and *AtPTB2*, respectively (Wang and Okamoto, 2009). Therefore, we refer to the more distantly related protein derived from *AT1G43190* as AtPTB3.

Auto- and cross-regulation of human *PTB*s have been shown to involve AS of their own pre-mRNAs and generation of NMD target transcripts (Grabowski, 2007). Furthermore, in a transcriptome-wide study, conserved AS events in *PTB* homologues from Arabidopsis and rice were identified (Wang and Brendel, 2006). Therefore, we first performed *in silico* analyses of AS patterns for Arabidopsis *PTB* homologues using transcript data available from TAIR (Swarbreck *et al.*, 2008) and ASIP (Wang and Brendel, 2006) databases. For all three *AtPTB*s, two splice variants are annotated with splice product SPI encoding full-length protein and SPII harbouring a PTC (Figure 1a). The expression of the respective mRNAs was experimentally validated by reverse transcription and PCR (RT-PCR) analyses from Arabidopsis seedlings (Figures 1b and S1). Using primer pairs covering the alternatively spliced regions, co-amplification of two PCR products corresponding to partial SPI and SPII sequences was achieved. Interestingly, the ratios of the co-amplified products indicated relatively low steady-state levels of SPII transcripts, which might be explained by NMD targeting resulting from the presence of a PTC in all SPII transcripts. This assumption was corroborated by a relative increase of SPII levels in the NMD-impaired mutant *lba1* (Yoine *et al.*, 2006) (Figure 1b,c).

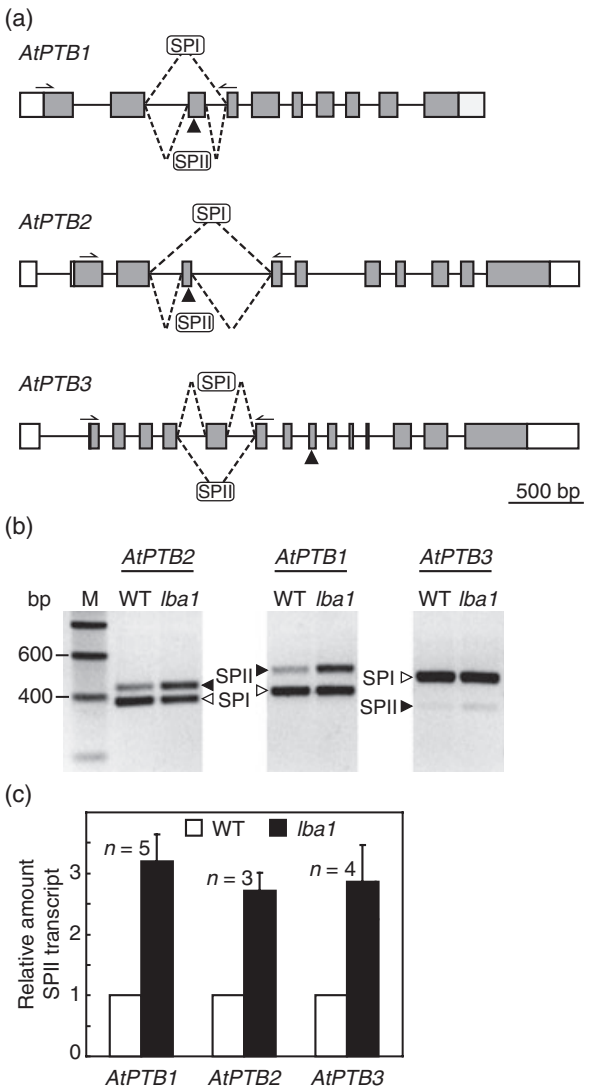

Figure 1. Arabidopsis *PTB* pre-mRNAs are alternatively spliced.
(a) Exon–intron organization of PTB-related genes *AtPTB1*, *AtPTB2* and *AtPTB3* from Arabidopsis. Exons and introns are represented by rectangles and lines, respectively. AS events, resulting in SPI and SPII variants, are indicated by broken lines. Protein coding regions of SPI transcripts are shaded in grey; black triangles refer to the positions of the premature termination codon (PTC) in SPII variants. For *AtPTB3*, cassette exon skipping in SPII leads to a frame-shift generating a PTC in a downstream constitutive exon. Arrows indicate positions of primer binding sites used for the RT-PCR analyses shown in (b).
(b) Detection of SPI and SPII mRNAs in samples from 14-day-old wild-type (WT) and NMD-impaired *lba1* seedlings via RT-PCR. Co-amplified PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining and UV illumination. M designates the size marker composed of DNA fragments in 200-bp increments.
(c) Increase of relative SPII levels for *AtPTB1*, *AtPTB2* and *AtPTB3* in *lba1* compared with WT seedlings. RT-PCR products shown in (b) were quantified using a bioanalyzer, and ratios were set to 1 for WT. Numbers of biological repeats, *n*; error bars, SD.

The relative increase of SPII levels in the *lba1* mutant provides evidence for NMD-targeting of those splicing variants. Thus, AS of *AtPTB* pre-mRNAs can be assumed to generate protein-encoding SPI transcripts, whereas SPII is degraded via NMD, and does not lead to protein production. To further test this hypothesis, fluorescence of reporter constructs containing the 5′ regions of either SPI or SPII transcripts fused in frame with the cDNA of enhanced green fluorescent protein (*EGFP*) (see Figure S2) were determined upon transformation into *Nicotiana benthamiana*. As expected, SPI-based constructs yielded robust reporter activity, whereas no significant fluorescence was detectable for SPII fusions (Figure S3). RT-PCR analysis of transformed leaves confirmed the expression of all fusion constructs on an mRNA level, whereas only SPI-based reporters resulted in protein production (Figure S3). Based on this experiment, translation from in-frame start codons, which are downstream of the PTCs, can be excluded, and SPI and SPII represent ON and OFF stages of gene expression, respectively.

Arabidopsis PTB homologues regulate AS of their own pre-mRNAs

In the next step, we sought to identify factors regulating the AS events of Arabidopsis *PTB* pre-mRNAs, and thus to gain insight into their functional relevance. Based on the regulatory features described for human PTBs (Grabowski, 2007), a role of Arabidopsis PTB proteins in AS control of their own pre-mRNAs was tested. Therefore, we co-expressed reporter constructs encompassing the 5′ genomic *PTB* sequences, including the alternatively spliced region fused to *EGFP* cDNA (pre-mRNA reporters, abbreviated as 'PRE') and full-length *PTB* cDNA constructs (Figure S2). The PRE reporter constructs can be spliced into SPI- and SPII-type transcripts, but they contain only a part of *PTB*, and are therefore not expected to result in a functional PTB protein. In contrast, full-length *PTB* cDNA constructs result in the accumulation of the respective PTB proteins (see below). In a previous report by Wang and Okamoto (2009), *AtPTB1* and *AtPTB2* were described as functionally redundant, with the latter one being more strongly expressed. Additionally, the high level of sequence conservation and equivalent AS patterns of *AtPTB1* and *AtPTB2* support their identity as close homologues. Therefore, we focussed our studies on reporters deduced from *AtPTB2* and the distinctly related *AtPTB3*. Expression of reporter constructs were analysed in transiently transformed *N. benthamiana* leaves by comparing the effect of co-expression of a control protein (Luciferase) versus PTB full-length proteins on two halves of the same leaf. The fluorescence level of the *AtPTB2*-PRE reporter was reduced to <10% by AtPTB2 and AtPTB1 proteins, whereas AtPTB3 only diminished reporter activity to ~60% relative to the control (Figure 2a). Immunoblot analysis with an EGFP-specific antibody revealed that reduced fluorescence levels were accompanied by diminished levels of EGFP fusion proteins (Figure 2b). Imperfect correlations between immunoblots and fluorescence values are based on the fact that protein levels were detected only in select samples, whereas quantitative reporter data were collected from many biological replicates. The observed reporter response might be explained by altered AS patterns, as splicing to SPI- but not to PTC-bearing SPII-mRNAs result in EGFP translation. To address this question, splicing patterns of the AtPTB2-PRE reporter were analysed. Under control conditions, the reporter was fully and exclusively spliced to SPI mRNA (Figure 2c). However, co-expression of AtPTB2 and AtPTB1 proteins changed splicing patterns to the formation of both SPI and SPII mRNAs, with the increased SPII levels being in

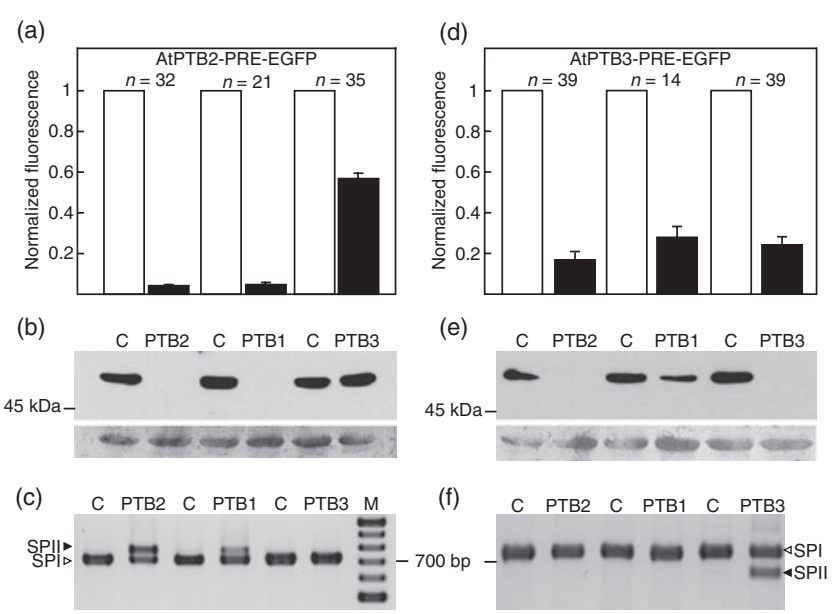

Figure 2. AtPTBs exert negative control on reporter constructs based on their own pre-mRNAs.
(a, d) Reporter constructs based on the pre-mRNA of *AtPTB2* (a) or *AtPTB3* (d) were transiently co-expressed with AtPTB proteins in *Nicotiana benthamiana* leaves, and fluorescence determined relative to a control protein (Luciferase, C). Numbers of biological repeats, *n*; error bars, SE.
(b, e) Immunoblot analysis of reporter proteins upon co-expression of AtPTBs or control protein. Upper panels show immunodetection of the fusion proteins with an EGFP-specific antibody; lower panels display amido black-stained membranes, with the large subunit of Rubisco as a loading control.
(c, f) RT-PCR analyses of AtPTB2-PRE-EGFP (c) and AtPTB3-PRE-EGFP (f) splicing under control conditions (C), and in the presence of various AtPTBs. SPI- and SPII-corresponding products are indicated, M designates the size marker. The primer binding positions are depicted in Figure S2.

line with reduced fluorescence. Interestingly, AtPTB3 protein did not alter *AtPTB2*-PRE reporter splicing, yet fluorescence was reduced, pointing to a splicing-independent regulatory mechanism. Comparable expression levels of all AtPTB proteins were confirmed by immunoblot analysis of tagged constructs (Figure S4).

Equivalent experiments were performed with an *AtPTB3*-pre-mRNA-based reporter (Figure 2d–f). All three AtPTB proteins similarly reduced reporter activity to 20–30%, but only in the presence of AtPTB3 were both SPI and SPII transcripts generated. As for *AtPTB2*, changes in splicing patterns were only observed in the presence of the corresponding protein (or, in the case of *AtPTB2*, additionally for the close homologue). However, splicing-independent negative regulation is also found for the other combinations.

The specificity of the observed effects was substantiated by co-expression of, and normalization to, a constitutive red fluorescent protein construct (DsRED). Furthermore, no AtPTB effect was observed for unfused EGFP and a previously described alternatively spliced reporter, based on transcription factor IIIA (Hammond *et al.*, 2009) (Figure S5). We also investigated the splicing of a reporter construct based on the 5′ genomic *AtPTB1* sequence in *N. benthamiana*, but only splicing to SPI was observed (Figure S6). This observation might be explained by interference of the reporter sequence context with splicing regulation (see also below). Furthermore, PRE reporters were also stably transformed into Arabidopsis, where, in the case of *AtPTB2* and *AtPTB3*, equivalent splicing patterns as those for the endogenous genes were observed (Figure S7), further substantiating the transferability of our reporter data. In contrast, the *AtPTB1*-PRE-EGFP reporter resulted in multiple splicing products, which is in line with the unexpected splicing behaviour in *N. benthamiana*.

Our reporter data suggested that elevated AtPTB protein levels result in an increased proportion of the corresponding pre-mRNAs being spliced to SPII mRNAs, whereas diminished AtPTB protein levels should yield less SPII transcripts. To test this hypothesis in a homologous system for the endogenous gene, we altered *AtPTB2* expression in stably transformed Arabidopsis plants by constitutive expression of the *AtPTB2* cds, or by introducing an *AtPTB2*-specific, artificial microRNA (amiRNA) construct. Analysis of total *AtPTB2* transcript levels confirmed the reduction and over-expression of *AtPTB2* mRNAs in several independent amiRNA- and overexpression lines, respectively (Figure S8). Subsequently, the relative proportions of SPII to total *AtPTB2* mRNAs derived from the endogenous gene were determined in wild-type and transformed plants. It is important to note that in the cds over-expression lines, *AtPTB2* mRNAs derived from the endogenous gene could be distinguished from transgene-derived transcripts because of their distinct 3′ untranslated regions (UTRs). Furthermore, all SPII mRNAs must have been derived from the endogenous gene, as in the over-expression lines no processing of the cds-based mRNAs to SPII transcripts was possible. Consistent with our model, downregulation of *AtPTB2* expression resulted in a marked decrease of relative SPII levels, whereas upon *AtPTB2* over-expression, elevated levels of SPII-type mRNAs were observed (Figure 3). To further investigate cross-regulatory effects, relative levels of SPII transcripts derived from *AtPTB1* were also analysed for the plants with altered *AtPTB2* expression. Interestingly, similar albeit less pronounced effects on *AtPTB1* SPII levels were observed, with a reduction in amiRNA lines and an increase upon over-expression of *AtPTB2* (Figure 3). Based on the relative changes of *AtPTB1* and *AtPTB2* SPII levels in the amiRNA lines, it can be assumed that under these conditions ~80 and ~20% of SPII production is caused by auto- and cross-regulation, respectively. In summary, for both the endogenous genes in Arabidopsis and the reporter constructs in *N. benthamiana*, a direct correlation between *AtPTB* expression and SPII production as a result of auto- and cross-regulation was established.

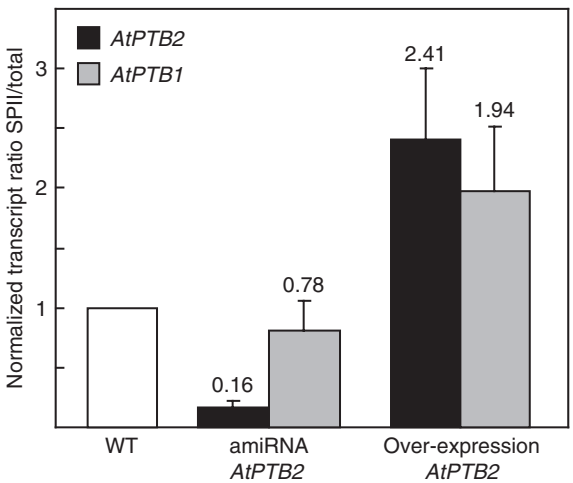

Figure 3. The accumulation of SPII-type mRNAs derived from *AtPTB2* and *AtPTB1* correlates with the total expression level of *AtPTB2* in Arabidopsis. Ratios of SPII to total transcripts derived from the endogenous *AtPTB2* and *AtPTB1* genes were determined by qPCR analysis of ~6-week-old plants. Reduced and increased *AtPTB2* levels relative to WT were achieved by constitutive expression of an artificial microRNA (amiRNA) and the *AtPTB2* cds, respectively (also see Figure S8). The ratio was set to 1 for the wild-type (WT). Data are mean values from eight and seven independent lines transformed with the amiRNA- and overexpression construct, respectively. Error bars represent SD.

Based on the markedly distinct splicing-regulatory effects of the three Arabidopsis PTB homologues, we were interested to explore the impact of a PTB protein from a non-plant species on expression of AtPTB-PRE reporters. Interestingly, co-expression of human PTB1 completely altered the splicing of AtPTB2- and AtPTB3-PRE reporters, yielding splice forms mainly based on intron retention (Figures 4a and S9), which resulted in reduced fluorescence because of the presence of PTCs (Figure 4b). The influence

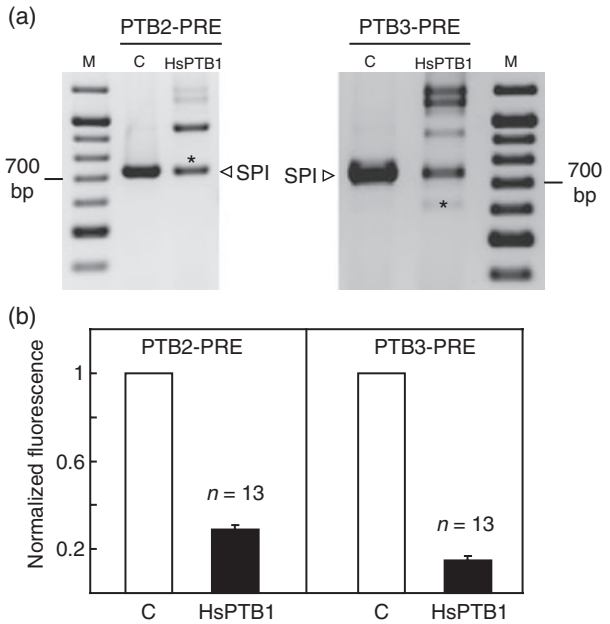

Figure 4. Human PTB alters splicing of *AtPTB* pre-mRNA-deduced reporter constructs.
(a) RT-PCR analyses of splicing patterns for reporter constructs based on the genomic 5′ region of *AtPTB2* and *AtPTB3* fused to EGFP in the presence of co-expressed control protein (C) or human *PTB* (HsPTB1). Asterisks indicate the expected positions of SPII mRNAs. Constructs were transiently transformed into *Nicotiana benthamiana*. M designates the size marker composed of DNA fragments in 100-bp increments up to 1000-bp and the top band corresponding to 1200-bp. Primer binding sites and sequences of splicing products are provided in Figures S2 and S9, respectively.
(b) Normalized fluorescence for the same construct combinations as described in (a). Fluorescence was analyzed 2 days after transient transformation, as prolonged *HsPTB1* expression resulted in severe leaf damage and general repression for the reference reporter. Numbers of biological repeats, *n*; error bars, SE.

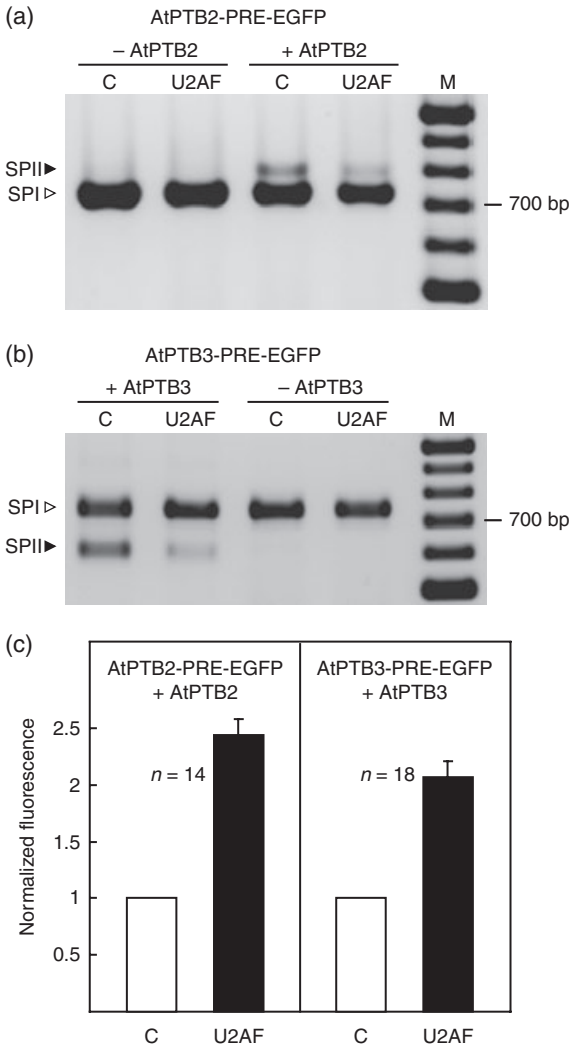

Figure 5. The splicing factor U2AF^{65} antagonizes AtPTB-mediated AS regulation.
(a) RT-PCR analysis of the effect of U2AF^{65} on *AtPTB2*-PRE-EGFP reporter splicing in the absence or presence of co-expressed AtPTB2 protein in *Nicotiana benthamiana*. C designates control sample with co-expressed luciferase. The co-expression of reporter and cds constructs was achieved by mixing *Agrobacterium* suspensions containing the various constructs in equal quantities, with the exception of the *AtPTB2* cds construct, which was diluted 1:1. M designates the size marker composed of DNA fragments in 100-bp increments.
(b) Equivalent experiment for an AtPTB3-PRE-EGFP reporter as shown for AtPTB2-PRE-EGFP in (a). Agrobacteria containing the different constructs were used in equal quantities for leaf infiltration.
(c) Normalized fluorescence levels of AtPTB2-PRE-EGFP and AtPTB3-PRE-EGFP in the presence of the respective AtPTB proteins and a control protein (C) or U2AF^{65} in *N. benthamiana* leaves. Co-expression of various constructs as described above. Numbers of biological repeats, *n*; error bars, SE.

of human PTB on the splicing of *AtPTB*-based reporters further supports the existence of variable as well as conserved target sequences and regulatory features of various PTB proteins.

To address the mechanism of AtPTB-mediated splicing regulation, we tested a possible role of the splicing auxiliary factor U2AF^{65}. Indeed, co-expression of *U2AF*^{65} counteracted the effect of both AtPTB2 and AtPTB3 on the splicing of the respective reporters, whereas in the absence of AtPTB, no change in reporter splicing was observed (Figure 5a,b). Reduced formation of SPII mRNAs upon co-expression of *U2AF*^{65} was also reflected by the de-repression of reporter activity (Figure 5c). Based on these data, AtPTB-mediated AS control appears to involve outcompeting U2AF^{65} binding, and thereby facilitates the generation of SPII transcripts.

Splicing-independent repression by Arabidopsis PTB homologues

Analyses of AtPTB-PRE reporter expression provided evidence that PTB-mediated negative control is not restricted to splicing regulation. To further investigate splicing-independent effects, we applied cDNA reporter constructs deduced from the fully spliced SPI mRNAs (Figure S2). All three AtPTB proteins similarly reduced reporter activity to ~50–60 and ~40% for *AtPTB2*- and

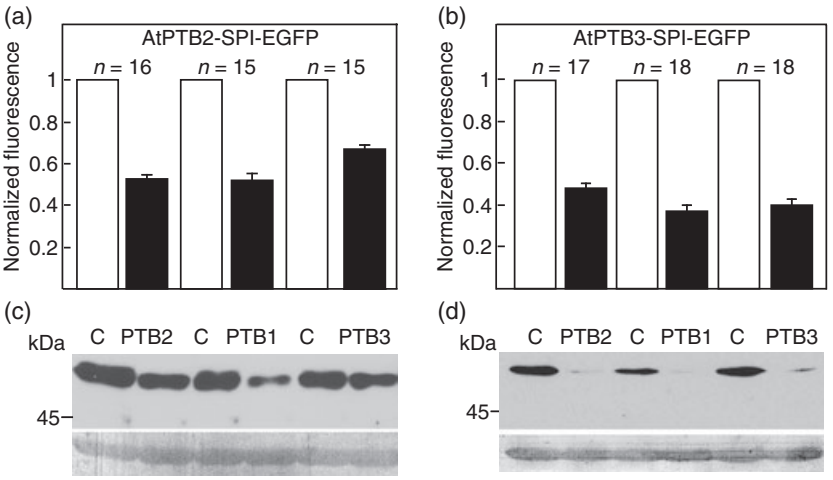

Figure 6. Splicing-independent repression of gene activity by AtPTBs.
(a, b) Impact of AtPTB proteins on the expression of reporters based on SPI variants for *AtPTB2* (a) and *AtPTB3* (b). Reporters and AtPTB proteins were transiently co-expressed in *N. benthamiana* and fluorescence values determined relative to a control (C). Numbers of biological repeats, *n*; error bars, SE.
(c, d) Immunoblot analysis of reporter proteins for samples corresponding to (a) and (b). Upper panels display immunosignals with an EGFP antibody, lower panels show amido black-stained Rubisco protein on the membranes as a loading control.

AtPTB3-SPI-based reporters, respectively (Figure 6a,b). Immunoblot analysis revealed that diminished fluorescence values were accompanied by reduced fusion protein levels (Figure 6c,d). Using RT-PCR and quantitative PCR, respectively, splicing and altered mRNA levels of the reporter transcripts upon AtPTB co-expression were excluded (Figure S10). From these data, it can be inferred that AtPTBs also act downstream of splicing control, such as in translational repression.

Arabidopsis PTB homologues localize to the nucleus, cytosol and P-bodies

Our finding that AtPTBs can perform regulatory tasks on different levels of gene expression could imply their localization in distinct cellular compartments. Therefore, we conducted *in vivo* localization studies using full-length proteins of AtPTB1, AtPTB2 and AtPTB3 N-terminally fused to EGFP. Co-localization studies with DsRed as a reference in Arabidopsis protoplasts showed that all three AtPTBs can be detected in the nucleus and the cytosol (Figure 7a–c), consistent with their role in splicing and translation control, respectively. Nuclear localization of AtPTBs was validated by co-localization with the exclusively nuclear marker NLS-DsRed (Figure S11). Interestingly, for approximately half of all protoplasts transformed with AtPTB1-EGFP or AtPTB2-EGFP (count given in Table S2), EGFP was also detected in small bodies dispersed within the cytosol (Figure S12). To clarify the identity of those bodies, co-localization experiments with established marker constructs showing similar fluorescence patterns were performed. Whereas no overlap with a peroxisomal marker construct was observed (data not shown), co-localization of AtPTB1/2-YFP with Decapping 1 (DCP1)-CFP (Xu *et al.*, 2006) identified these structures as P-bodies (Figure 7d,e). As P-bodies have been described as highly dynamic and variable structures (Weber *et al.*, 2008), we confirmed P-body localization of AtPTB fusions by co-transformation with another established P-body marker based on the exoribonuclease *XRN4* (Weber *et al.*, 2008) (Figure S13). Interestingly, co-expression of *DCP1*-CFP increased the number of cells displaying P-body localization for AtPTB1- and AtPTB2-EGFP fusions, in addition to nuclear and cytosolic localization (counts given in Table S2). This observation might be explained by the highly dynamic nature of P-bodies, the formation of which depends on the levels of their constituents. The absence of AtPTB fluorescence signals in P-bodies of some protoplasts is likely to be attributed to either expression below the detection limit (as AtPTBs, in contrast to DCP1-CFP, also localize to the nucleus and cytosol) or cell-specific variations. Furthermore, upon co-expression of DCP1-CFP, partial co-localization with AtPTB3-YFP was observed in a substantial fraction of transformed cells (Figure 7f; Table S2). However, in comparison with *AtPTB1*- and *AtPTB2*-based constructs, AtPTB3-EGFP accumulated only to relatively low levels in P-bodies, and therefore was only visible in larger structures. Importantly, the co-transformation of DCP1-CFP did not alter the localization of cytosolic YFP, further supporting the specificity of the reference construct (Figure S14; Table S2). Expression of all reporter fusions was driven by the strong CaMV35S promoter, which might lead to massive protein accumulation and, subsequently, mis-localization of the respective fusion proteins. Therefore, *AtPTB2-YFP* was also expressed under the control of the *AtPTB2* promoter (Figure S15). Again, AtPTB2-YFP was detected in the cytosol, nucleus and P-bodies, speaking against a localization artifact arising from CaMV promoter-driven expression.

Next, we extended our localization studies from protoplasts to stably transformed Arabidopsis plants. Consistent with the previous results, all AtPTB fusions localized to the nucleus and cytosol of epidermal and guard cells (Figure 8). Additionally, for AtPTB1- and AtPTB2-EGFP, fluorescence signals were observed in bodies located within the cytosol. Interestingly, AtPTB3-EGFP again showed a variant localization pattern compared with reporters for the two other

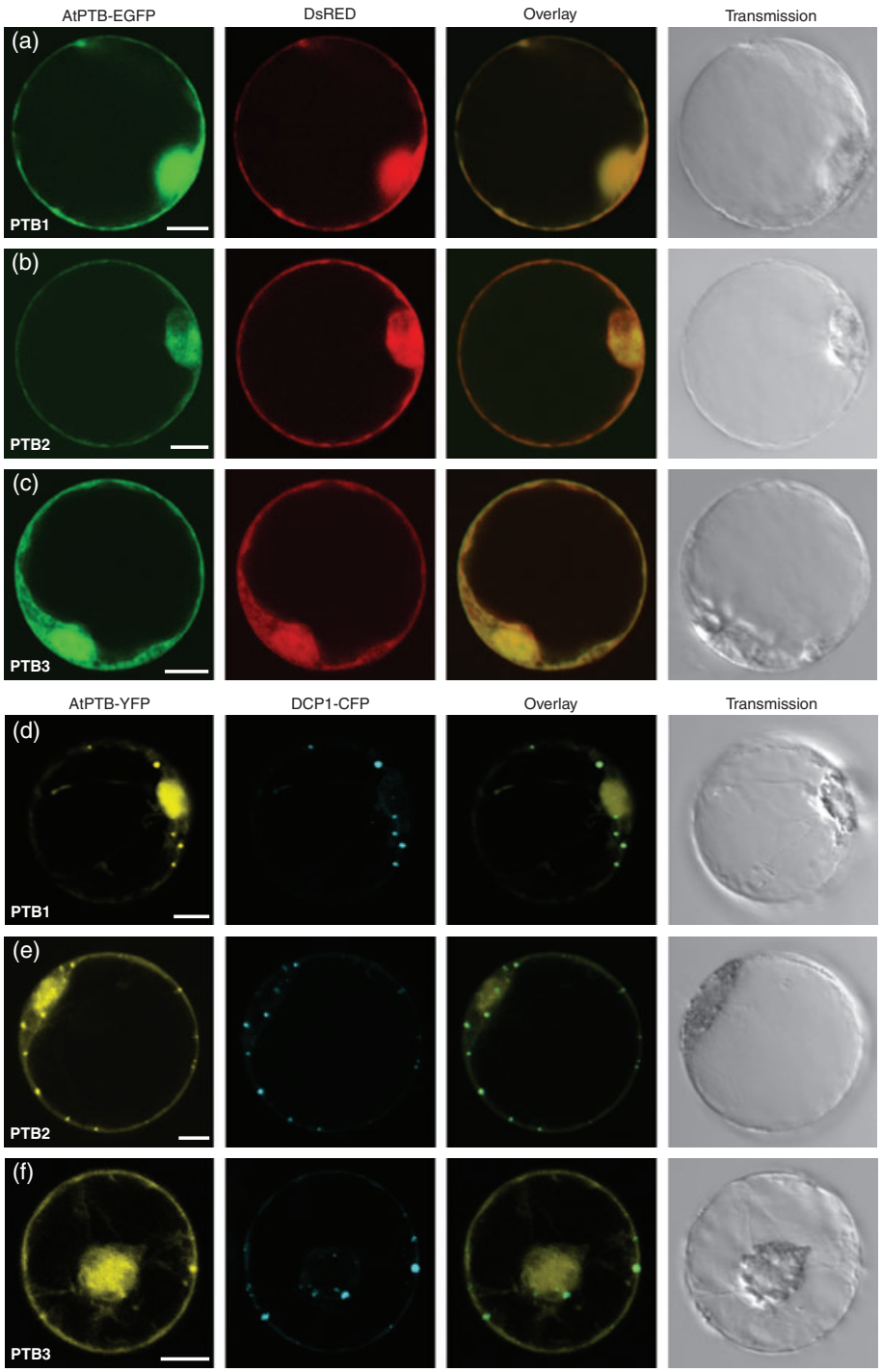

Figure 7. Subcellular localization of AtPTB reporter fusions in the nucleus, cytosol and processing bodies in living Arabidopsis protoplasts.
(a–c) Reporter constructs encoding full-length proteins of *AtPTB1* (a), *AtPTB2* (b) and *AtPTB3* (c) N-terminally fused to EGFP were transiently co-expressed with DsRED in Arabidopsis protoplasts. Images show signals for EGFP and DsRED fluorescence, overlay and transmission light for representative protoplasts. Scale bars: 10 μm.
(d–f) *In vivo* co-localization of AtPTB-YFP fusions and the processing body marker DCP1-CFP. Images show signals for YFP, CFP, overlay and transmission light for representative protoplasts.

homologues with less well-defined structures, albeit not homogenously distributed fluorescence within the cytosol (Figure 8). Identical localization patterns were observed for cells within the hypocotyl of stably transformed AtPTB-EGFP lines (Figure S16). In summary, our *in vivo* reporter data established the subcellular localization of AtPTBs in the

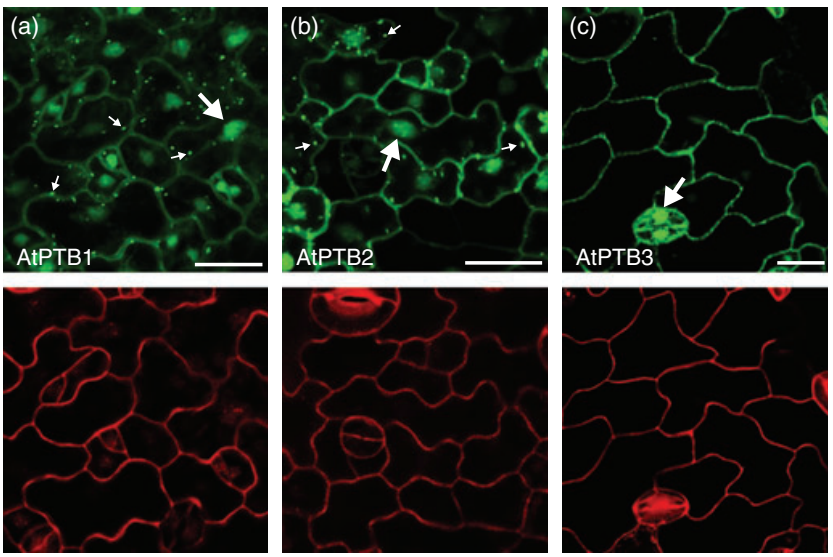

Figure 8. *In vivo* localization of AtPTB-EGFP fusion proteins in stably transformed Arabidopsis plants.
Subcellular localization of AtPTB1-EGFP (a), AtPTB2-EGFP (b) and AtPTB3-EGFP (c) in leaves of 5-day-old seedlings. Upper panel shows EGFP fluorescence of representative samples as detected by confocal laser scanning microscopy. Leaves were stained with propidium iodide to visualize cell boundaries (lower panel). With EGFP-specific settings, no signals in wild-type Arabidopsis leaves were observed. Small and large arrows point exemplarily to fluorescent cytosolic bodies and nuclei, respectively. Scale bars: 20 μm.

nucleus, cytosol and in cytoplasmatic structures resembling P-bodies.

DISCUSSION

Coupling of AS and NMD controls the expression of Arabidopsis PTB homologues

In this report, we provide evidence that all three Arabidopsis *PTB* homologues generate two types of splicing variants, one of which leads to full-length protein production, whereas the other is subjected to NMD. Furthermore, a transcriptome-wide survey of AS in plants (Wang and Brendel, 2006) and an analysis of annotated transcripts (data not shown) revealed the existence of equivalent splice variants for PTB homologues from rice, pointing to an evolutionary conservation of these AS events and their functional implications. Previous studies have established PTCs, 3′–UTR-positioned introns, and long 3′–UTRs as major NMD-triggering *cis*-elements of mRNAs in plants (Kertesz *et al.*, 2006; Kerenyi *et al.*, 2008). According to a transcriptome-wide analysis of splicing variants, the coupling of AS and NMD holds an enormous potential as a plant gene control mechanism (Wang and Brendel, 2006). This was further corroborated by the fact that in NMD-impaired Arabidopsis mutants, an increase of numerous, predicted NMD target transcripts was observed (Hori and Watanabe, 2005; Arciga-Reyes *et al.*, 2006; Yoine *et al.*, 2006). For example, Palusa and Reddy (2010) demonstrated the extensive coupling of AS and NMD for SR genes, with approximately half of the 53 PTC-containing splicing variants derived from 13 SR genes being NMD targets. However, only in a few instances has the coupling of AS and NMD as a means of plant gene regulation been experimentally tested. One intriguing example is the regulatory circuit of the Arabidopsis RNA-binding proteins *AtGRP7* and *AtGRP8* that are, similar to Arabidopsis *PTB* homologues, subject to auto- and cross-regulation via AS-coupled NMD (Schoning *et al.*, 2008). Other examples in plants include riboswitch-mediated gene control (Bocobza *et al.*, 2007; Wachter *et al.*, 2007), as well as the coordination of transcription factor IIIA expression with ribosomal protein L5 levels (Hammond *et al.*, 2009). Based on this growing number of instances, it is becoming evident that NMD, besides its mRNA surveillance function in the elimination of aberrant transcripts, originated from erroneous gene expression, plays a fundamental role in plant gene regulation. Hence, the more widespread role of NMD in gene regulation as well as the inter-kingdom conservation of NMD mechanisms (Kerenyi *et al.*, 2008) highlights the similarities between NMD functions and pathways in different eukaryotes. Interestingly, profound differences might exist in the subcellular localization of NMD, as a recent report suggested a role of the nucleolus in plant NMD (Kim *et al.*, 2009).

Mode of action of Arabidopsis PTB homologues: splicing-dependent and downstream regulatory mechanisms

Our experiments established a splicing-regulatory function for AtPTBs that can alter AS of their own pre-mRNAs. Furthermore, for AtPTB1 and AtPTB2, splicing control was found to be extended to their close homologues. As elevated AtPTB protein levels changed AS in favour of a PTC-containing NMD target, we propose a model of negative auto- and cross-regulation. Interestingly, similar splicing-regulatory circuits exist for human PTBs (Wollerton *et al.*, 2004; Boutz *et al.*, 2007a; Spellman *et al.*, 2007), which have also been described as regulators of global AS patterns in developmental processes (Boutz *et al.*,

2007a). This splicing regulatory function allows feedback control of PTB protein production and, even more intriguingly, provides a basis for interlinking the expression of various PTB homologues with distinct features. Specifically, a switch between two related human PTB homologues was shown to involve AS-coupled NMD and, subsequently, reprogrammed AS patterns in the development from neuronal progenitor cells to neurons (Boutz *et al.*, 2007b; Grabowski, 2007). Based on the numerous analogies between plant and human *PTB*s, it is tempting to speculate that the three *PTB* homologues in Arabidopsis might similarly play a role as regulators of AS programmes.

PTB pre-mRNAs from Arabidopsis and human are alternatively spliced by control of a cassette exon (Figure 1; Grabowski, 2007). From studies in animals, it is known that PTB typically suppresses the inclusion of an exon adjacent to its binding site (Sawicka *et al.*, 2008). Accordingly, in humans, the PTC-containing splicing variants preferentially formed in the presence of elevated PTB protein levels are lacking the cassette exon, the inclusion of which is required to encode the full-length protein (Grabowski, 2007). Whereas an equivalent mechanism is found for *AtPTB3*, in the case of *AtPTB1* and *AtPTB2*, the NMD target variant is generated by the retention of the cassette exon. A genome-wide mapping of PTB-RNA interactions revealed that PTB binding near a constitutive splice site typically leads to exon inclusion, whereas binding in the proximity of an alternative site induces exon skipping (Xue *et al.*, 2009). Therefore, we can conclude that AtPTB3 most probably binds close to the alternative 3′ splice site upstream of the cassette exon, whereas AtPTB1 and AtPTB2 are expected to bind close to the constitutive 3′ splice site, downstream of the respective cassette exons. Furthermore, we could demonstrate that AtPTB-mediated splicing regulation is counteracted by $U2AF^{65}$, which further supports similar modes of actions of PTB proteins from animals and plants. However, analysis of reporter construct splicing also revealed remarkably variant splicing regulatory features among the three AtPTBs, as well as compared with human PTB. This selectivity of AtPTB-mediated splicing control might also hint at a putative role of AtPTBs as splicing regulators with distinct target pre-mRNAs. Importantly, different, albeit not necessarily mutually exclusive, models have been proposed for PTB-mediated splicing control in animals (Spellman and Smith, 2006), and further studies will be needed to gain deeper insight into the functioning of PTB homologues in plants.

In addition to their splicing regulatory function, PTB proteins are known to control other processes such as translation from internal ribosomal entry sites (Sawicka *et al.*, 2008). Furthermore, Boutz *et al.* (2007b) reported that, in the presence of PTB protein, properly spliced mRNA of the homologue nPTB does not lead to nPTB protein accumulation, indicating the existence of an additional gene control mechanism. In line with this hypothesis, our reporter studies also revealed a splicing-independent component of AtPTB-mediated negative regulation. This repression had no significant effect on the steady-state levels of target mRNAs, suggesting instead that AtPTBs can control the level of translation. As a possible mechanism, AtPTBs could retract their target mRNAs from the pool of translated transcripts, which would be in agreement with the subcellular localization of AtPTBs in P-bodies (see below). Ultimately, multi-layer control allows for tight regulation of PTB expression, and further highlights the importance of co-ordinating activities of various PTB homologues in plants and animals.

Arabidopsis PTB homologues localize to the nucleus, cytosol and P-bodies

In line with the regulatory tasks performed by AtPTBs, our *in vivo* localization studies revealed their presence in different compartments. Whereas nuclear localization would be claimed for a splicing factor, the identification of AtPTBs as a P-body component was not expected. P-bodies refer to cytoplasmic foci with functions in mRNA storage, translational repression, and mRNA degradation (Sheth and Parker, 2003). In yeast, it has been demonstrated that upon P-body association, mRNAs are not necessarily subjected to subsequent decay, but can also re-enter the polysomal fraction, representing a transient translational arrest (Brengues *et al.*, 2005). The characterization of Arabidopsis homologues of decapping complex proteins and their localization in cytoplasmic foci (Xu *et al.*, 2006; Goeres *et al.*, 2007; Xu and Chua, 2009) provided evidence that P-bodies also exist in plants, even though many questions about their functions remain to be addressed. Based on our findings that AtPTBs can localize to P-bodies and act as translational repressors, we propose that mRNA-PTB complexes might be sequestered in P-bodies for translational arrest. Interestingly, a recent report by Gaddy *et al.* (2010) described the localization of PTB in punctate cytoplasmic sites of human cells upon cytomegalovirus infection, indicating that P-body localization might be a more general feature of PTBs. Previous studies have also highlighted the dynamic and flexible nature of P-bodies (Weber *et al.*, 2008), which might explain the variations observed for AtPTB localization.

In conclusion, our work provides insight into the gene regulatory potential of Arabidopsis PTB homologues, which can control AS, and presumably the translation of target mRNAs. The expression of *AtPTB*s is interlinked by auto- and cross-regulatory circuits, establishing a basis for distinct cellular functions. In light of these findings, future studies need to identify novel PTB targets and thereby illuminate the biological implications of this multifunctional protein group in plants.

EXPERIMENTAL PROCEDURES

Plant cultivation

Arabidopsis thaliana ecotype Columbia-0 was grown on soil at ~20°C and 60% humidity in a growth chamber (16-h light at ~120 μE/8-h dark). For sterile growth, Arabidopsis seedlings were cultivated for up to 2 weeks on basal MS medium complemented with 2% sucrose under the conditions stated above. *N. benthamiana* plants were grown on soil for 4–5 weeks in a glasshouse.

Oligonucleotides and cloning procedures

A list of all oligonucleotides and a description of cloning procedures are provided in Table S3 and Appendix S1, respectively.

RNA isolation and RT-PCR

Total RNA was extracted from ~100 mg of plant tissue using the Universal RNA Purification kit (Roboklon, http://www.roboklon.de), including an on-column DNaseI treatment performed according to the manufacturer's instructions. Subsequently, ~5 μg of total RNA was subjected to reverse transcription with dT_{20} using AMV Reverse Transcriptase Native (Roboklon), as described in the supplier's protocol. For RT-PCR analyses of PTB and EGFP mRNAs, DnaseI-treated RNAs were included as negative controls. RT-PCR products were separated and visualized using Agarose gel electrophoresis and ethidium bromide staining or a Bioanalyzer (Agilent, http://www.chem.agilent.com). RT-PCR products were directly sequenced (LGC Genomics, http://www.agowa.de) or were first cloned into pGEM-T vector (Promega, http://www.promega.com). Binding sites of primers used for RT-PCR are indicated in Figures 1, S1 and S2.

Quantitative PCR

Quantitative PCR was performed using the Biorad CFX384 real-time PCR system and MESA GREEN qPCR Mastermix Plus (Eurogentec, http://www.eurogentec.com). Serial dilutions of the templates were conducted to determine primer efficiencies. Reactions were performed in triplicate, and amplification products were examined by melting curve analysis. Data were analyzed using the relative standard curve method, and expression was normalized relative to the housekeeping gene *AT1G13320* (PP2A catalytic subunit) or, for transiently transformed *N. benthamiana* leaves, to a co-expressed reporter.

Plant transformation methods

Arabidopsis protoplasts derived from a heterotrophic cell culture line of ecotype Columbia-0 were transformed with 2.2 μg plasmid DNA for each construct, and transformation was performed as described in Schutze *et al.* (2009). After transformation, protoplasts were incubated for 2 days in the dark and then analysed by microscopy. Arabidopsis was stably transformed by a floral-dip method, described previously (Clough and Bent, 1998). For transient transformation of *N. benthamiana*, an Agrobacteria-based leaf infiltration assay was used (Wachter *et al.*, 2007), as outlined in Appendix S1.

Quantitative reporter fluorescence measurements

Proteins were extracted from transiently transformed *N. benthamiana* leaves collecting ~100 mg of tissue from each leaf half, followed by extraction with 300 μl buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v.v) Tween 20, 0.1% (v.v) β-mercaptoethanol]. Upon centrifugation, supernatants were used to measure EGFP and DsRED fluorescence values with a fluorometer (TriStar; Berthold, http://www.berthold.com). EGFP was excited at 478–492 nm and detected at 515–525 nm; the normalization control DsRED was excited at 525–535 nm and detected at 595–605 nm.

In vivo localization studies by confocal laser scanning microscopy

Arabidopsis protoplasts or 5-day-old seedlings grown on moistened filter paper were analysed with a confocal laser scanning microscope (TCS SP2 AOBS; Leica, http://www.leica-microsystems.com). Fluorescent proteins were analysed with the following settings for excitation and emission, respectively: EGFP (488, 600–657 nm), YFP (514, 770–800 nm), CFP (405, 566–617 nm), DsRED (561, 822–850 nm). For cell wall staining, Arabidopsis seedlings were incubated for 30 min in 50 μM propidium iodide, and were then subsequently washed with water. Propidium iodide fluorescence was excited and detected at 488 and 822–850 nm, respectively. Digital images and artworks were processed with Adobe CS3 (Adobe, http://www.adobe.com).

Immunoblot analysis

Protein extraction was performed as described for fluorescence measurements. SDS-PAGE and immunoblots were conducted according to standard protocols using 10 μg of total proteins for each sample. As primary antibodies, anti-EGFP from rabbit (Invitrogen, http://www.invitrogen.com) and anti-FLAG from mouse (Sigma, http://www.sigmaaldrich.com) were used, followed by chemiluminescence detection (Super Signal West Dura; Pierce, http://www.piercenet.com), following the manufacturer's protocol.

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SUPPORTING INFORMATION

The following supplementary material is available for this article online:

Figure S1. Binding sites of primers used for RT-PCR and sequences of amplification products corresponding to SPI and SPII variants of AtPTBs.

Figure S2. Schematic representation of AtPTB-EGFP reporter constructs.

Figure S3. SPII fusion constructs show strongly reduced protein expression in comparison with SPI reporters for all three AtPTBs.

Figure S4. AtPTB proteins accumulate to comparable levels upon transient expression in *Nicotiana benthamiana*.

Figure S5. AtPTB proteins do not affect activities of control reporter constructs.

Figure S6. Analysis of AtPTB1-based PRE-EGFP reporter splicing.

Figure S7. Reporter constructs based on AtPTB pre-mRNAs show equivalent splicing patterns as endogenous PTB genes in stably transformed Arabidopsis plants.

Figure S8. AtPTB2 mRNA levels in stably transformed Arabidopsis lines with constitutive expression of either an AtPTB2-directed amiRNA or the AtPTB2 cds.

Figure S9. Sequences of cloned human PTB1 and splicing products generated upon co-expression of human PTB1 and AtPTB2- or AtPTB3-pre-mRNA reporters.
Figure S10. RT-PCR and qPCR analyses of AtPTB-SPI-based reporter transcripts upon co-expression of AtPTB proteins.
Figure S11. Nuclear localization of AtPTB-EGFP fusions is corroborated by co-transformation with the exclusively nuclear marker NLS-DsRED.
Figure S12. In a significant proportion of all transformed Arabidopsis protoplasts, AtPTB-EGFP fusions are also found in cytoplasmic bodies.
Figure S13. Co-localization of AtPTB-YFP fusion proteins with XRN4-CFP in processing bodies of Arabidopsis protoplasts.
Figure S14. The P-body marker DCP1-CFP does not affect localization of unfused YFP in the cytosol and nucleus.
Figure S15. AtPTB2-YFP expressed under the control of the AtPTB2 promoter also localizes to the nucleus, cytosol and processing bodies.
Figure S16. *In vivo* localization of AtPTB-EGFP fusions in cells within the hypocotyl of stably transformed Arabidopsis plants.
Table S1. Comparison of human PTB1 and related proteins from Arabidopsis.
Table S2. Counts of cells exhibiting visible, cytoplasmic bodies in Arabidopsis protoplasts.
Table S3. Sequences of DNA oligonucleotides.
Appendix S1. Supporting experimental procedures.
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LARGE-SCALE BIOLOGY ARTICLE

Polypyrimidine Tract Binding Protein Homologs from *Arabidopsis* Are Key Regulators of Alternative Splicing with Implications in Fundamental Developmental Processes[W]

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Alternative splicing (AS) generates transcript variants by variable exon/intron definition and massively expands transcriptome diversity. Changes in AS patterns have been found to be linked to manifold biological processes, yet fundamental aspects, such as the regulation of AS and its functional implications, largely remain to be addressed. In this work, widespread AS regulation by *Arabidopsis thaliana* Polypyrimidine tract binding protein homologs (PTBs) was revealed. In total, 452 AS events derived from 307 distinct genes were found to be responsive to the levels of the splicing factors PTB1 and PTB2, which predominantly triggered splicing of regulated introns, inclusion of cassette exons, and usage of upstream 5′ splice sites. By contrast, no major AS regulatory function of the distantly related PTB3 was found. Dependent on their position within the mRNA, PTB-regulated events can both modify the untranslated regions and give rise to alternative protein products. We find that PTB-mediated AS events are connected to diverse biological processes, and the functional implications of selected instances were further elucidated. Specifically, *PTB* misexpression changes AS of *PHYTOCHROME INTERACTING FACTOR6*, coinciding with altered rates of abscisic acid–dependent seed germination. Furthermore, AS patterns as well as the expression of key flowering regulators were massively changed in a PTB1/2 level-dependent manner.

INTRODUCTION

Eukaryotic precursor mRNAs (pre-mRNAs) are subject to extensive co- and posttranscriptional processing, which represents an essential step in the generation of mature, translation-competent mRNAs. Besides its fundamental role in constitutive gene expression, pre-mRNA processing also increases transcriptome complexity by producing distinct mRNA variants from one type of pre-mRNA, thereby providing an additional layer of gene regulation. In particular, alternative pre-mRNA splicing (AS) and alternative 3′ end processing have been found to expand transcriptome diversity enormously in both animals (Mangone et al., 2010; Nilsen and Graveley, 2010) and plants (Reddy, 2007; Hunt, 2011; Syed et al., 2012). While alternative 3′ end processing generates mRNAs of variable lengths, an even more diverse outcome can be achieved by AS via removal of variable single or multiple intronic regions. The resulting splicing variants can vary in their coding regions and in the presence of *cis*-regulatory elements, both of which can have important functional implications.

Among the different types of AS, exon skipping, intron retention, and the use of alternative 5′ and/or 3′ splice sites are most common (Black, 2003; Reddy, 2007). In human, 95% of all intron-containing genes were estimated to be affected by AS (Pan et al., 2008), and recent comprehensive transcriptome analyses based on deep sequencing data revealed a wide extent of AS in plants as well (Filichkin et al., 2010; Lu et al., 2010; Zhang et al., 2010; Marquez et al., 2012). Applying high-throughput RNA sequencing (RNA-seq) techniques, 42 to 61% of all multiexon genes from *Arabidopsis thaliana* (Filichkin et al., 2010; Marquez et al., 2012) and 33 to 48% of all rice (*Oryza sativa*) genes (Lu et al., 2010; Zhang et al., 2010) were found to be associated with AS events. Despite its wide distribution and its huge regulatory potential, many fundamental questions, in particular concerning the control of AS decisions as well as the biological implications of the vast majority of events, remain to be elucidated. AS can, on the one hand, lead to an increase in proteome diversity; however, only few instances in plants have been studied in detail, such as *XBAT35* in *Arabidopsis* (Carvalho et al., 2012). While a comparative analysis of plant splicing variants suggested a minor role of AS in proteome expansion (Severing et al., 2009), the recent identification of many additional splicing variants might lead to a different conclusion. On the other hand, splicing variants can differ in their repertoire of *cis*-regulatory elements with functions in mRNA metabolism and transport. Importantly, many splicing variants display features such as premature termination codons (PTCs) and/or long 3′ untranslated regions (UTRs)

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that are expected to trigger the degradation of these splice forms via the eukaryotic RNA surveillance mechanism nonsense-mediated decay (NMD; Chang et al., 2007; Nicholson et al., 2010). Importantly, NMD not only functions in the clearance of erroneous transcripts originating, for example, from mistakes in transcription or processing, but also enables gene regulation via degradation of AS variants (Lareau et al., 2007). Examples from plants include RNA binding proteins, such as the Gly-rich RNA binding proteins At-GRP7 and -GRP8 (Staiger et al., 2003; Schöning et al., 2008), Ser/Arg-rich (SR) proteins (Kalyna et al., 2003; Palusa and Reddy, 2010), and Polypyrimidine tract binding protein (PTB) homologs (Stauffer et al., 2010). These proteins can act in auto- and cross-regulatory negative feedback loops by triggering splicing of their own pre-mRNA or that of related proteins into an NMD target variant. Interestingly, a previous analysis of transcript features indicated that 43 and 36% of all AS events from *Arabidopsis* and rice, respectively, produce potential NMD targets (Wang and Brendel, 2006). A major role of NMD in targeting AS products was further substantiated by a recent study showing that out of 270 selected genes, 32% generated splicing variants with elevated levels in NMD mutants (Kalyna et al., 2012).

AS decisions are defined by the splicing code, an interaction network of *trans*-acting splicing factors and *cis*-regulatory elements (Chen and Manley, 2009). *Cis*-elements within the pre-mRNA function not only as binding sites for spliceosomal components and regulatory factors, but can also play a more active role in directing splicing, as illustrated by eukaryotic riboswitches (Bocobza et al., 2007; Wachter et al., 2007; Wachter, 2010). Widespread splicing regulatory functions have been assigned to members of the groups of SR and heterogeneous ribonucleoprotein (hnRNP) proteins that often act in a combinatorial manner. While most of our current knowledge on AS regulation is based on animal systems, previous work has provided evidence for the presence of SR (Barta et al., 2008; Reddy and Shad Ali, 2011) and hnRNP proteins (Wachter et al., 2012) and their roles within splicing control in plants. Furthermore, a function of the nuclear cap binding protein complex in controlling AS events in *Arabidopsis* has been demonstrated (Raczynska et al., 2010). Given that several aspects of AS, such as its prevalent types, have been shown to differ between animals and plants (Reddy, 2007), a thorough analysis of the currently ill-defined plant splicing code is of central importance for our understanding of this process (Reddy et al., 2012).

Earlier studies suggested that SR and hnRNP proteins generally act as splicing activators and repressors, respectively. However, more recent work suggested that the splicing regulatory activity can vary for different binding sites as well as for sets of combinatorially acting factors. One example for position-dependent splicing is constituted by the hnRNP protein PTB (Xue et al., 2009; Llorian et al., 2010), an in animals, well-characterized regulator of AS that binds to pyrimidine-rich motifs within pre-mRNAs (Sawicka et al., 2008; Wachter et al., 2012). Evidence has been provided that PTB exploits various mechanisms for AS control, including competing with U2 auxiliary factor 65 in binding to the pre-mRNA (Saulière et al., 2006), looping of RNA regions (Spellman and Smith, 2006), and interference with splicing factor interactions required for exon or intron definition (Izquierdo et al., 2005; Sharma et al., 2005). In animals, a switch in expression from PTB to its neuronal homolog nPTB was shown to reprogram AS patterns and coincides with neuronal development (Boutz et al., 2007). While regulated splicing networks as basis of fundamental biological programs have so far not been characterized in plants, numerous studies supported the occurrence of specific AS patterns linked to certain tissues, development, and stress responses in plants (Palusa et al., 2007; Simpson et al., 2008; Filichkin et al., 2010; Zenoni et al., 2010). Furthermore, important roles of AS control in the regulation of the circadian clock (Sanchez et al., 2010; Staiger and Green, 2011; James et al., 2012) and flowering time (Deng et al., 2010) have been reported.

Interestingly, homologs of PTB proteins are also found in plants. In pumpkin (*Cucurbita maxima*), the PTB homolog RBP50 is translocated from source to sink tissues and was shown to be a component of a phloem-mobile ribonucleoprotein complex (Ham et al., 2009). In *Arabidopsis*, three genes encoding proteins with homology to mammalian PTBs have been identified. Two of these *Arabidopsis* PTB homologs have been suggested to be involved in pollen germination (Wang and Okamoto, 2009), but the underlying molecular basis has not been addressed. While the proteins encoded by *At3g01150* (PTB1) and *At5g53180* (PTB2) are closely related, the protein encoded by *At1g43190* (PTB3) exhibits a quite low level of sequence similarity to the other two. All three PTB homologs from *Arabidopsis* have been shown to generate two types of splice variants of which one encodes the full-length protein, whereas the alternative variant contains a PTC and is subject to degradation via NMD (Stauffer et al., 2010). Based on their ability to alter AS of their own pre-mRNAs in favor of the PTC-containing transcript variant, a model of negative auto- and cross-regulation was proposed (Stauffer et al., 2010; Wachter et al., 2012). Interestingly, comparable regulatory circuits have also been described for the mammalian PTB homologs (Wollerton et al., 2004; Boutz et al., 2007). While these findings provided evidence for the splicing regulatory potential of At-PTBs, a possible existence of further splicing regulation targets as well as the overall functional implications of this group of proteins in *Arabidopsis* remained unresolved.

In this study, we generated a set of transgenic *Arabidopsis* lines having either up- or downregulated PTB levels and subjected these to transcriptome-wide AS analyses. Based on opposite splicing ratio changes in plants with elevated and decreased PTB levels, 452 AS events were identified as potential direct PTB1/2 splicing regulation targets. These AS events comprised mainly alternative 5′ splice site selection, intron retentions, and cassette exons. Independent experimental testing of selected instances confirmed their authenticity. Furthermore, specific as well as redundant splicing regulatory activities of the two closely related proteins PTB1 and PTB2 were established, while no evidence for a major role of PTB3 in splicing control was found. Intriguingly, PTB1/2-regulated AS events are linked to genes with diverse biological functions, and a critical role of some of these AS processes in seed germination and flowering time control was revealed.

RESULTS

Generation of At-*PTB* Misexpression Lines

For the transcriptome-wide identification of potential targets of PTB-mediated splicing regulation, transgenic *Arabidopsis* lines with altered levels of these proteins were generated. *Arabidopsis* mutants with elevated PTB levels were obtained by transformation of Columbia-0 wild-type plants with constructs harboring the coding sequences (cds) of At-*PTB1* (OE1), At-*PTB2* (OE2), or At-*PTB3* (OE3) under control of the constitutive cauliflower mosaic virus promoter. Importantly, use of cds-based constructs prevented the previously described feedback regulation via AS (Stauffer et al., 2010). Downregulation of *PTB* expression was achieved by the transformation of wild-type plants with artificial microRNA (amiRNA) constructs, targeting either the three *PTBs* individually (ami1, ami2, and ami3) or the two closely related homologs *PTB1* and *PTB2* simultaneously (ami1&2; see Supplemental Table 1 online). The amiRNA knockdown approach was chosen as previous work had indicated that single T-DNA mutants in At-*PTB1* and At-*PTB2* have no visible phenotype, while the respective double mutant seemed to be inviable (Wang and Okamoto, 2009).

At least 10 individual T0 generation plants for each construct were analyzed for their *PTB* steady state transcript levels (see Supplemental Figure 1 online). Subsequently, individual lines with minimum and maximum *PTB* transcript levels were selected for further analyses, and alterations in *PTB* expression were confirmed in the following generations (Figure 1A). Relative to the wild-type, *PTB* overexpression resulted in an ~40- and ~15-fold higher level of *PTB1* and *PTB2* transcripts, respectively, while for *PTB3*, only a maximum approximately twofold increase was found. In line with the previously described cross-regulation between PTB1 and PTB2 (Stauffer et al., 2010), strong overexpression of one of these homologs resulted in decreased transcript levels of the other. By contrast, the modest overexpression of *PTB3* did not significantly reduce transcript levels of *PTB1* and *PTB2* and also had no effect on the corresponding protein levels (see below). Upon expression of amiRNAs targeting single *PTBs*, transcript levels of the respective targets were reduced to a minimum of ~30 to 40% in comparison to the wild-type (Figure 1A). The amiRNA construct for simultaneous knockdown of *PTB1* and *PTB2* resulted in a reduction of both mRNAs to ~40% of the wild-type transcript level.

To further characterize the misexpression lines, PTB protein levels were analyzed using affinity-purified polyclonal antibodies. Immunoblot analyses of samples from *Nicotiana benthamiana* leaves transiently expressing Flag-tagged At-PTB1, -PTB2, and

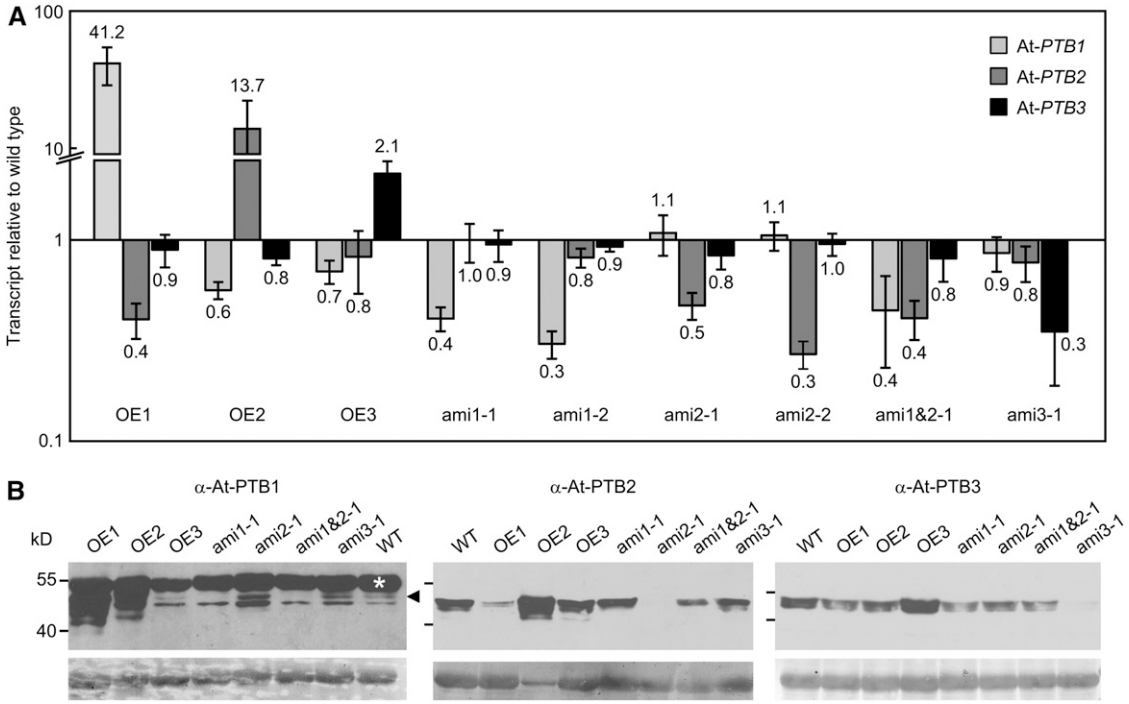

Figure 1. Altered At-PTB Transcript and Protein Levels in Overexpression and amiRNA Lines.

(A) Relative levels of *PTB1*, *PTB2*, and *PTB3* transcripts in either overexpression (OE) or amiRNA (ami) lines for all three At-PTBs, as determined by reverse transcription and quantitative PCR from 10-d-old seedlings. Values are calculated relative to a reference transcript and normalized to wild-type levels. Displayed are mean values (n = 3 to 6) ± SD.

(B) Immunoblot analyses of At-PTB1, -PTB2, and -PTB3 protein levels of samples equivalent to those described in **(A)**. Top panels show immune signals with purified At-PTB–specific antibodies, and bottom panels show amidoblack staining of membranes as loading control. For each sample, 15 μg of total protein was loaded, except for the OE2 sample on the PTB2 blot, for which only 3 μg total protein was loaded. For the PTB1 immunoblot, the white asterisk indicates an unspecific cross-reaction with RBCL protein, and the arrowhead points at the specific PTB1 signal. WT, the wild-type.

-PTB3 yielded strong and specific immune signals in case of the At-PTB2 and At-PTB3 antibodies (see Supplemental Figure 2 online). For the At-PTB1 antibody, an overall weaker signal and cross-detection of the closely related protein At-PTB2 was observed. Analyzing *Arabidopsis* seedlings, PTB1 was barely detectable in the wild-type, but accumulated to high levels in the respective overexpression line (Figure 1B; see Supplemental Figure 3 online). Strong overexpression of PTB2 again resulted in its cross-detection by the At-PTB1 antibody, which was not unexpected regarding the homology of the two proteins. However, a slightly different migration behavior of the two proteins was revealed by extended gel runs, which is in agreement with the calculated molecular weights of 43.6 and 46.9 kD for PTB1 and PTB2, respectively (see Supplemental Figure 3 online). Besides the major immune signal for PTB1 in the OE1 line, several weaker bands corresponding to slightly smaller proteins were detected, which might be due to degradation products and/or posttranslational modifications. Importantly, PTB1 immune signals were not detectable in the ami1 and ami1&2 lines, but, in line with the cross-regulatory potential, showed increased intensity in the ami2 sample. Equivalent results were obtained for PTB2 protein signals, as detected with an At-PTB2–specific antibody (Figure 1B). The same sample set was also analyzed with an At-PTB3–specific antibody, which gave rise to a broad signal at ~50 kD (calculated size: 48.2 kD) in wild-type seedlings (Figure 1B). This signal was strongly reduced in the ami3 line, while a moderate increase in the OE3 seedlings was observed. No major alterations of the PTB3 level in the other lines were found, providing evidence that PTB3 expression does not critically depend on PTB1 and PTB2 levels in seedlings. Estimation of PTB2 and PTB3 protein amounts based on the immune signals for the corresponding overexpression samples revealed an ~9- and 1.5-fold increase, respectively. Specificity of the immune signals was confirmed by analyzing single T-DNA insertion lines for all three *PTBs*, revealing a complete loss of the respective PTB bands (see Supplemental Figures 4 and 5 online). Furthermore, analysis of PTB2 and PTB3 protein levels in the misexpression lines at rosette stage showed that changes persisted during development (see Supplemental Figure 6 online). Interestingly, while PTB3 protein levels in seedlings were not altered upon misexpression of *PTB1* and *PTB2*, we found decreased and increased PTB3 levels in rosette leaves upon *PTB2* overexpression and in the ami1/2 lines, respectively (see Supplemental Figure 6 online). This finding suggests that the cross-regulation between the PTBs varies in a developmental manner. In summary, the immunoblot analyses revealed that PTB protein levels in the misexpression lines were successfully altered, making these lines adequate tools for downstream splicing studies.

Transcriptome-Wide Splicing Studies Reveal a Widespread Regulatory Role of At-PTBs

To identify PTB splicing regulatory targets, RNA-seq analyses of wild-type and *PTB* misexpression lines at seedling stage were performed. The resulting reads (see Supplemental Table 2 online) were mapped to the *Arabidopsis* genome (TAIR10 version), followed by the deduction of information on AS, which led to the identification of 26,076 AS events in total (Figure 2A). We found the usage of alternative 3′ splice sites (alt 3′, 40.6%) to be the most abundant AS type, followed by intron retention (29.8%) and alternative 5′ splice site choice (alt 5′, 21.8%). Finally, only 7.8% of all events were derived from alternative inclusion or skipping of an exon. By contrast, previous RNA-seq–based analyses of AS in *Arabidopsis* identified intron retention to be the most abundant type of AS (Filichkin et al., 2010; Marquez et al., 2012). However, an approximately twofold higher frequency of alt 3′ compared with alt 5′ usage as well as exon skipping being the least abundant among the four basic types of AS analyzed was also reported (Marquez et al., 2012). The varying frequencies of AS types between these studies can be attributed to differences in the biological material, RNA-seq procedures, and read alignments as well as the computational pipelines for defining novel splicing variants.

Analysis of read coverage for the *PTBs* confirmed specific changes in their transcript levels for the corresponding misexpression lines (see Supplemental Figure 7 online). For each AS event, ratios of the two splicing variants were analyzed in pairwise tests, by comparing wild-type, amiRNA, and OE samples and assigning P values to indicate significant changes (see Supplemental Data Set 1 online). We first strictly filtered for events significantly changed in comparison of the amiRNA versus the corresponding OE lines ($P < 0.005$). Subsequent filtering restricted the candidate list to AS events that were also changed in the comparison of wild-type and amiRNA lines, excluding events that only changed upon PTB overexpression and therefore could represent an artifact caused by the excessive supply of a splicing factor. Furthermore, events showing ratio changes in the same direction relative to the wild-type for both amiRNA and OE lines were not further considered. Applying these filter criteria to the ami1&2, OE1, OE2, and wild-type data sets resulted in the identification of 452 AS events being regulated by the closely related PTB1 and PTB2 proteins (Figure 2A). Interestingly, different frequencies of AS types for the PTB1/2-dependent events, in comparison to all AS events, were observed. While cassette exons and alt 5′ events were strongly overrepresented among the PTB1/2-regulated targets, an almost sevenfold decrease in the fraction of alt 3′ events was found. Intriguingly, applying the same filter criteria to the data sets of the corresponding *PTB3* misexpression lines yielded only five AS events derived from four different genes (see Supplemental Data Set 2 online). Independent testing confirmed PTB3-dependent changes in the AS ratio for only two of the five events (see Supplemental Figure 8 online), suggesting that PTB1 and PTB2, but not PTB3 have widespread AS regulatory functions.

Our analysis revealed that certain AS types were overrepresented among the PTB1/2-regulated AS events compared with all detected ones, and we next analyzed the direction of AS change. Downregulation of PTB1 and PTB2 triggered the retention of introns in 77% of all cases from this AS type, while 86% of all regulated cassette exons showed a shift toward the exon skipping version (Figure 2B). For alt 5′ events, PTB1/2 knockdown caused a preferential use of the downstream 5′ splice site, while there was no trend for the few detected PTB1/2-dependent alt 3′ events. Mapping of the AS event positions relative to the TAIR10 representative gene model revealed that

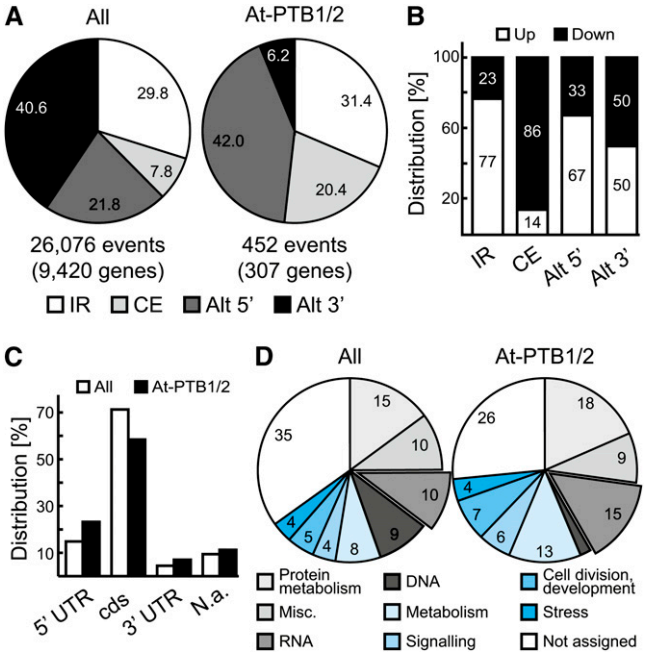

Figure 2. Transcriptome-Wide Identification of At-PTB Splicing Regulation Targets.

(A) Relative frequencies of different AS types for all detected AS events (left) and PTB1/2-regulated events (right). Total numbers of events and associated genes are indicated. alt 5′/3′, alternative 5′/3′ splice site; CE, cassette exon; IR, intron retention.

(B) Direction of splicing change for all At-PTB1/2–regulated AS events in the ami1&2-1 seedlings compared with the wild-type. "Up" and "Down" indicate a relative increase and decrease of the longer splicing variant, respectively.

(C) Frequencies of positions for all and the PTB1/2-regulated AS events in the UTRs and cds of the corresponding TAIR10 representative mRNAs as well as events that could not be implemented (N.a.).

(D) GO term analysis for all genes with a GO assignment and the PTB1/2 regulation targets. Relative proportion of genes with PTB1/2-regulated AS events belonging to GO term "DNA" is 2%.

58, 23, and 7% of the regulated events resided within the cds, 5′ UTR, and 3′ UTR, respectively (Figure 2C; see Supplemental Table 3 online). Compared with all detected events, this represents a higher fraction of UTR positions for the PTB1/2-dependent instances. Furthermore, sequence analyses of the associated splicing variants indicated that 72.3% of the regulated AS events generated at least one transcript form harboring NMD-eliciting features (see Supplemental Table 3 online). Gene ontology (GO) term analysis for the loci associated with PTB1/2-regulated AS events revealed interconnections with diverse biological functions (Figure 2D; see Supplemental Data Set 3 online). Comparing the frequencies of the different GO terms revealed a distinct overrepresentation of the term "RNA" among the PTB1/2 regulation targets relative to all genes, which was most pronounced for the subcategory "RNA processing" (see Supplemental Data Set 3 online). This finding suggests the existence of regulatory networks among genes involved in RNA metabolism with a role of PTB-dependent AS. The GO term "DNA" was clearly underrepresented among the PTB1/2 regulation targets, which can be ascribed to the large number of transposons and transposon-related functions within this GO term group.

To validate our findings of PTB-controlled AS events, representative examples for the different types of AS were selected for independent testing. Out of the 10 analyzed cassette exon events, nine showed reciprocal changes in the proportion of exon inclusion when comparing splicing patterns in the ami1&2-1 and the corresponding OE lines (Figure 3; see Supplemental Figures 9 and 10 online). For *At1g07350*, a pronounced ratio change in the ami1&2, but not in the OE1/2 lines, relative to the wild-type was detected, suggesting that for this event, PTB1 and PTB2 levels might already be saturating in wild-type seedlings. Two of the cassette exon candidates (*At1g07350* and *At1g19800*) were selected based on a previous estimate of exon inclusion rates (data not shown) but did not meet our first stringent filter criterion (the P value was ~0.02 in the comparison of line ami1&2-1 versus OE PTB1/2). Nonetheless, both events could be verified experimentally, suggesting that PTB1/2 regulate even more AS events than estimated based on our stringent filtering. Nine of the tested cassette exons showed an increased ratio of skipping to retention in the ami1&2-1 sample, whereas *At2g34357* showed the opposite direction of change. Furthermore, while downregulation of PTB1/2 had similar quantitative effects for most events, more variation was observed for the OE lines, suggesting that PTB-dependent cassette exons respond differently to varying levels of PTB proteins in vivo. To exclude a general perturbation of AS upon *PTB* misexpression, splice form patterns for the previously characterized cassette exon event within *At1g72050* (Hammond et al., 2009) were determined (Figure 3B).

Our transcriptome-wide splicing studies indicated that the At-PTBs not only regulate cassette exon splicing, as predominantly reported in animals, but also numerous intron retention and alt 5′ events. Therefore, events belonging to these AS-type categories were selected for independent, experimental verification, confirming their authenticity with reciprocal changes in the *PTB1/2* misexpression lines (Figure 4; see Supplemental Figures 10 and 11 online). Alt 3′ events were by far least abundant among the PTB1/2-regulated events; nevertheless, two of these events were selected for independent experimental validation. Splicing variant ratios for wild-type and *PTB1/2* misexpression seedlings were unaltered in case of *At2g13790*, but slightly changed for *At2g43330* (see Supplemental Figures 10 and 12 online).

Subsequently, we determined whether the two close homologs PTB1 and PTB2 act redundantly in splicing regulation and if some of the identified events are also responsive to the distinct homolog PTB3. Therefore, splicing variant ratios of previously described PTB-dependent AS events were determined in seedlings expressing amiRNAs against *PTB1* or *PTB2* (Figure 5A; see Supplemental Figure 13 online). A number of the tested AS events did not show any alteration in the single amiRNA lines compared with the wild-type, suggesting a compensation of the knockdown of either PTB1 or PTB2 by its close homolog. A second category of events showed the same direction of change in the single amiRNA lines as was observed for simultaneous knockdown of PTB1 and PTB2, however, with less pronounced quantitative changes, indicating a redundant function of the two PTBs in the

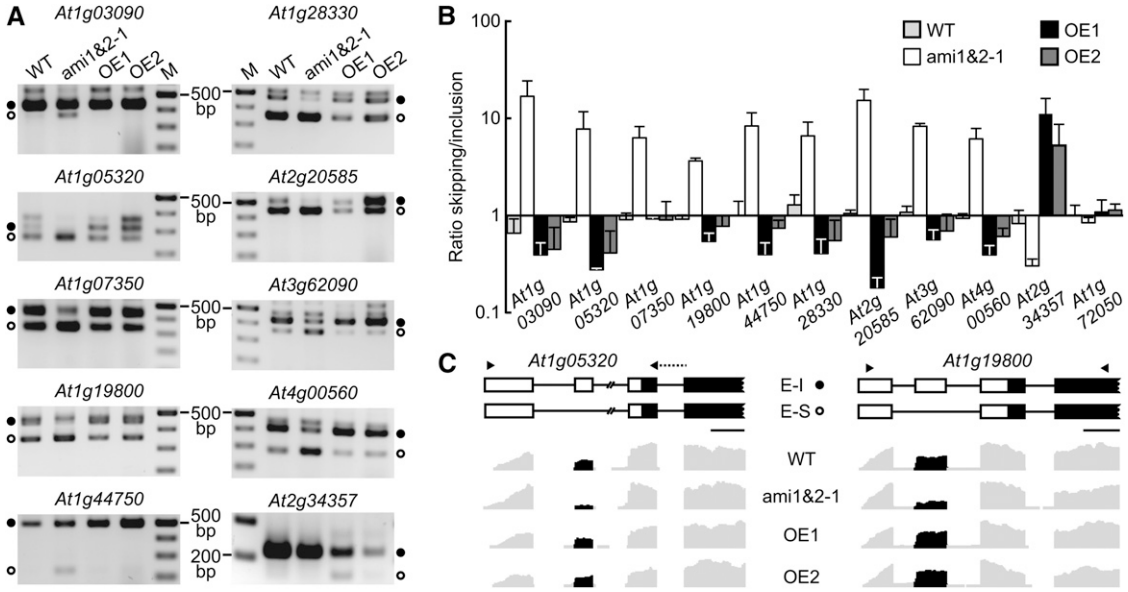

Figure 3. Regulation of Cassette Exon Splicing by At-PTB1 and -PTB2.

(A) RT-PCR analysis of cassette exon splicing ratios for 10 selected genes in seedlings of the genotypes displayed at the top. Black and open circles indicate the bands corresponding to exon inclusion and skipping, respectively. All major bands have been identified by sequencing (see Supplemental Figure 10 online). M designates marker consisting of DNAs in 100 bp increments. WT, the wild-type.

(B) Quantitative analysis of splicing ratios for the candidates shown in **(A)** and a PTB-independent cassette exon event derived from *At1g72050* as negative control using a bioanalyzer. Displayed are mean values (n = 3 to 5) with SD.

(C) Partial gene models depicting the alternatively spliced regions of *At1g05320* and *At1g19800* and corresponding coverage plot areas showing RNA-seq results of the indicated samples. Exon and introns are displayed as boxes and lines, respectively, with black boxes representing cds. Arrows and arrowheads indicate primer binding positions used for coamplification of splicing variants in **(A)** and **(B)**; black lines are scales corresponding to 100 bp length. Coverage plot reads within alternatively spliced region are shown in black, and other reads are in gray.

regulation of these events. For several events in this category, a stronger effect upon PTB2 knockdown was found, suggesting a more dominant regulatory role of this splicing factor compared with PTB1 under the tested conditions. Intriguingly, the cassette exon events associated with the genes *At1g07350* and *At2g20585* showed pronounced opposite ratio changes upon single knockdown of PTB1 or PTB2. These findings strongly suggest that not only the absolute levels of PTB1 and PTB2, but also the ratio of the two proteins have important implications in AS control. Furthermore, analysis of the splicing ratios for selected PTB1/2-regulated AS events showed no or only weak AS changes in the *PTB3* misexpression lines (Figure 5B).

AS of *PIF6* mRNA Is Regulated by At-PTBs and Correlates with Altered Seed Germination

To gain insight into the biological implications of At-PTB–controlled AS events, the list of splicing regulation targets was examined for candidates that have previously been studied with respect to their function, especially regarding the roles of individual splicing variants. One particularly interesting example represented *At3g62090* that encodes PHYTOCHROME INTERACTING FACTOR6 (PIF6) and harbors a PTB1/2-regulated cassette exon event. Studies based on constitutive overexpression of the individual *PIF6* splicing variants had revealed that the exon skipping, but not the inclusion variant, can reduce primary seed dormancy (Penfield et al., 2010). Analysis of *PIF6* AS in different tissues of flowering wild-type plants showed a relative increase of the exon skipping variant in buds compared with leaf tissue (Figure 6A). Downregulation and overexpression of PTB1 and/or PTB2 perturbed *PIF6* mRNA splicing in opposite directions and compromised or fully prevented the tissue-specific AS switch. Furthermore, PTB-linked deregulation of *PIF6* splicing was found in dry seed (Figures 6B and 6C).

To uncover a possible link between *PTB* expression and germination behavior, germination rates of after-ripened seed in the absence or presence of exogenous abscisic acid (ABA) were determined (Figures 6D and 6E; see Supplemental Figure 14 online). While no pronounced difference in seed germination was detectable in the absence of exogenous ABA, elevated and diminished germination rates for the ami1&2-1 and the OE1 lines, respectively, were observed in assays on ABA-containing medium. Interestingly, the changes in germination rates correlated with alterations in AS of the *PIF6* mRNA, which were most pronounced upon simultaneous knockdown of PTB1 and PTB2 as well as overexpression of PTB1. The increased germination rate coincided with a relative increase of the exon skipping variant in the ami1&2-1 plants, being in line with the described germination stimulating effect upon overexpression of this splicing variant. Single knockdown of PTB1 also slightly increased the seed germination rate in the presence of ABA at the early time points (Figure 6E; see Supplemental Figure 14

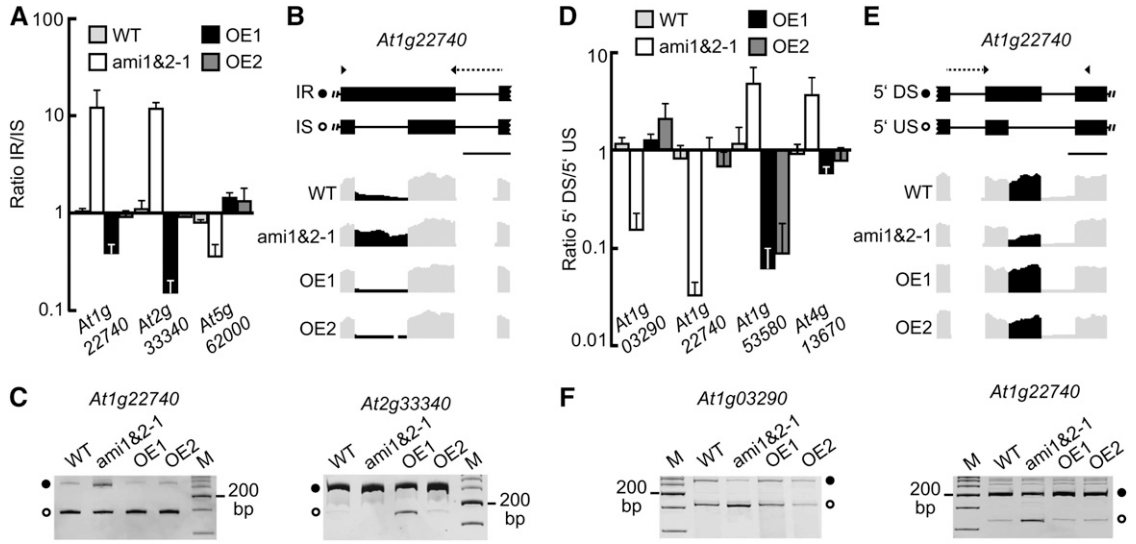

Figure 4. Regulation of Intron Retention and Alternative 5′ Splice Site Choice by At-PTB1 and -PTB2.

(A) Quantitative analysis of transcript ratios for selected intron retention cases in seedlings of genotypes as indicated using a bioanalyzer. Displayed are mean values (n = 3 to 5) with SD. WT, the wild-type; IR, intron retention; IS, intron splicing.

(B) Partial gene model depicting the alternatively spliced region of *At1g22740* and corresponding coverage plot areas showing RNA-seq results of the indicated samples.

(C) RT-PCR analysis of transcript ratios for *At1g22740* and *At2g33340* in seedlings of genotypes as displayed. Black and open circles indicate the bands corresponding to intron retention and splicing, respectively. M, marker consisting of DNAs in 100 bp increments.

(D) Quantitative analysis of transcript ratios for selected alternative 5′ splice site cases as described in **(A)**. DS/US, downstream/upstream splice site.

(E) Partial gene model depicting the region with the two alternative 5′ splice sites for *At1g22740* and corresponding coverage plot areas showing RNA-seq results of the indicated samples.

(F) RT-PCR analysis of transcript ratios for *At1g03290* and *At1g22740* in seedlings of displayed genotypes. Black and open circles indicate the bands corresponding to transcripts derived from the downstream and upstream 5′ splice site, respectively.

online), yet had no effect later on. Consistent with our finding of weak or no ABA-dependent germination effects for the ami1 and ami2 plants, only moderate AS changes for *PIF6* in seedlings (Figure 5A) and dry seed (see Supplemental Figure 14D online) were detected. Overexpression of PTB3 also affected seed germination, yet no opposing effect upon amiRNA knockdown of this homolog was found, speaking against a direct regulatory role. The AS event within *PIF6* leads to inclusion/skipping of an exon within the cds without altering the reading frame and therefore is expected to result in the production of two protein variants. Using a PIF6-specific antibody, we could demonstrate that constitutive expression of the splicing variants in *N. benthamiana* and *Arabidopsis* results in accumulation of proteins of the expected sizes (see Supplemental Figure 14E online), while the PIF6 protein levels in wild-type samples were below the detection limit. Based on these data, the proteins encoded by the *PIF6* splicing variants might accumulate to similar extent in vivo.

To test if At-PTB–mediated AS control of the *PIF6* pre-mRNA is caused by a direct protein/RNA interaction, electrophoretic mobility shift assays (EMSAs) were performed. Using an RNA fragment encompassing the *PIF6* cassette exon and flanking intronic sequences, direct binding of recombinant Thioredoxin-At-PTB2 fusion protein was detected (Figure 7). Control reactions with identical amounts of an unrelated protein of similar size as PTB2 and containing the same Thioredoxin tag did not result in an RNA shift. Interestingly, the analyzed RNA probe contained several polypyrimidine stretches positioned within or downstream of the regulated cassette exon, and further studies need to clarify their putative functions in PTB binding.

At-PTBs Control AS and Expression of Fundamental Flowering Regulator Genes

Splicing of the pre-mRNAs from the flowering regulator genes *FLOWERING LOCUS K* (*FLK*) and *FLOWERING LOCUS M* (*FLM*) was found to be PTB dependent as well. Simultaneous knockdown of *PTB1* and *PTB2* triggered inclusion of an intron within the 5′ UTR of the *FLK* mRNA, while overexpression of *PTB1* had the opposite effect (Figures 8A and 8B). FLK positively regulates flowering by repressing the expression of the flowering repressor *FLOWERING LOCUS C* (*FLC*; Lim et al., 2004; Mockler et al., 2004), and a link between *FLK* pre-mRNA splicing and the expression of *FLC* has been reported previously (Deng et al., 2010). Specifically, an increase of the *FLK* intron retention variant reduced the level of functional FLK protein (Deng et al., 2010), thus resulting in elevated levels of *FLC* expression. Therefore, an increased level of *FLC* expression in the ami1&2-1 plants, displaying a higher rate of FLK intron retention, was anticipated. Intriguingly, the opposite change of *FLC* levels was found, with the *FLC* transcript being undetectable upon PTB1/2 knockdown and strongly elevated in the *PTB1* overexpression plants compared with the wild-type (Figure 8B). Furthermore, an

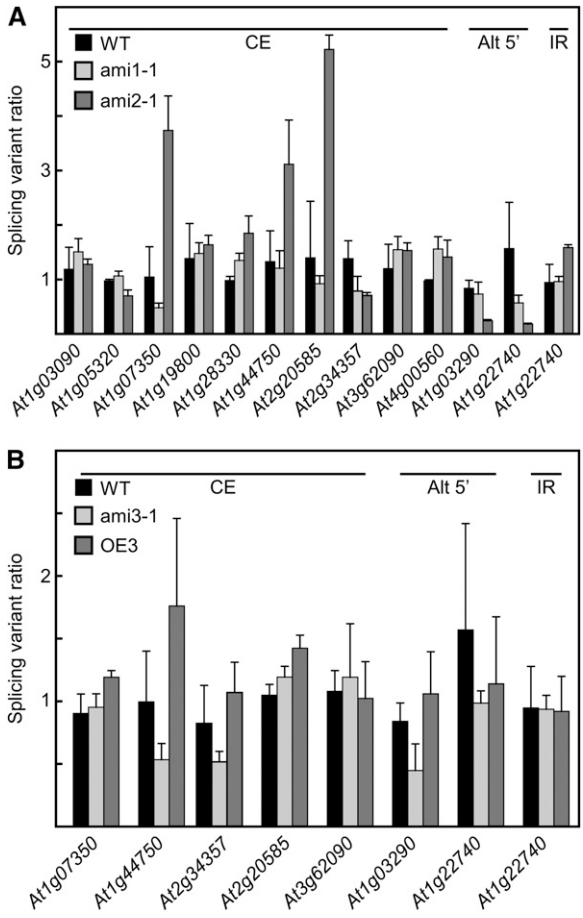

Figure 5. Specific and Redundant Splicing Regulatory Activities of At-PTBs.

(A) Splicing variant ratios in seedling tissues of wild-type (WT), ami1-1-, or ami2-1-expressing plants. Data are mean values (n = 3) with SD of bioanalyzer quantitation. Displayed are examples for cassette exons (CE), alternative 5′ splice site choice (Alt 5′), and intron retention (IR).

(B) Splicing variant ratios of selected PTB1/PTB2-dependent splicing events in *PTB3* misexpression lines. Data are mean values (n = 3) with SD of bioanalyzer quantitation. Other details as described in **(A)**.

altered AS pattern was observed for the pre-mRNA of the *FLC*-like clade member *FLM*, of which one splicing variant has also been reported to encode a floral repressor (Ratcliffe et al., 2001). In the ami1&2-1 plants, an increased level of the mRNA containing the upstream exon was detected (Figure 8B), while levels of the downstream cassette exon splicing variant as well as the coverage within some introns were reduced (Figures 8A and 8B). Thus, the downregulation of *FLC* transcript levels upon knockdown of *PTB1* and *PTB2* is accompanied by AS-mediated down- and upregulation of the floral activator *FLK* and a repressor-encoding *FLM* splicing variant, respectively. Flowering time analysis of *PTB* misexpression lines under short- and long-day conditions revealed that the onset of flowering is unchanged compared with the wild-type (see Supplemental Figure 15 online), suggesting that the changes in positive and negative floral regulators might compensate for each other and/or that additional control mechanisms occur. To assess if the massive increase in *FLC* transcript levels in the OE1 lines might translate into elevated levels of functional FLC protein, read coverage plots were inspected for the occurrence of putative RNA processing variants. Furthermore, four and seven independent, full-length *FLC* cDNA sequences from wild-type and OE1 samples, respectively, were cloned and sequenced. These analyses supported only the occurrence of the representative transcript variant in TAIR10 (*At5g10140.1*). In the absence of any other inhibitory mechanism, this variant should lead to production of functional FLC protein. Moreover, assessment of the levels of the previously reported *COOLAIR* antisense RNAs (Swiezewski et al., 2009) in the *PTB* misexpression lines revealed similar trends of changes as the corresponding sense transcript (see Supplemental Figure 16 online).

DISCUSSION

Regulation of Complex AS Profiles in *Arabidopsis*

Our work identified a few hundred AS events to be specifically altered in their splicing variant ratio output in response to changed levels of the splicing factors PTB1 and PTB2 (Figure 2). Importantly, all of the detected splicing variants were already present in wild-type seedlings, thus not representing artifacts generated by reduced or elevated PTB levels. Recent studies applying high-throughput sequencing techniques revealed that AS is widespread in *Arabidopsis* (Filichkin et al., 2010; Marquez et al., 2012) and AS patterns have been found to be linked to fundamental processes, including abiotic stress responses (Filichkin et al., 2010) and regulation of the circadian clock (Sanchez et al., 2010; James et al., 2012). From these findings, not only a high diversity of AS in plants, but also a prominent role of AS regulation linked to diverse cellular signals can be inferred. While previous studies in plants have mainly addressed the AS regulation for single pre-mRNAs, such as auto- and cross-regulation of At-*GRPs* (Staiger et al., 2003; Schöning et al., 2008), this work provides evidence for complex splicing networks linked to single splicing factors. Considering the huge number of plant splicing regulators, such as the factors belonging to the groups of SR (Barta et al., 2008; Reddy and Shad Ali, 2011) and hnRNP proteins (Wachter et al., 2012), a wide scope of AS in modulating gene expression can be anticipated. Intriguingly, the identified PTB-regulated AS events are associated with genes involved in diverse biological processes, and the functional relevance of selected events has been confirmed in the context of seed germination and flowering control.

Comparing the PTB1/2-dependent to all detected AS events, an overrepresentation of alternative 5′ splice site choice and cassette exons has been found. However, this preference might be linked to these particular splicing factors instead of representing a general bias of regulated AS events. Furthermore, the events found to be regulated by PTB1/PTB2 are located both within the UTRs and the cds and can thus contribute to proteome diversity as well as regulation via UTR-borne signals. Given that a significant fraction of the PTB1/2-regulated transcripts contains NMD-eliciting features, a role of PTBs in gene regulation via coupling of AS and NMD can be anticipated.

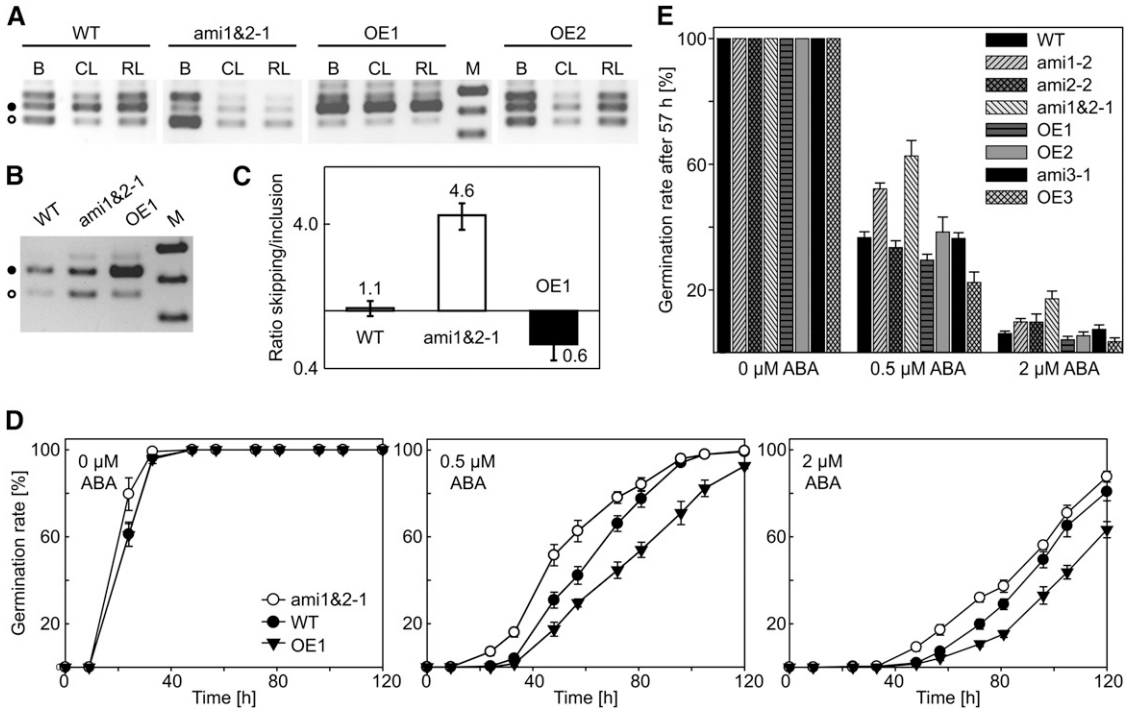

Figure 6. Altered At-PTB Levels Correlate with Changes in *PIF6* Splicing and ABA-Dependent Seed Germination.

(A) RT-PCR analysis of *PIF6* splicing patterns in the wild-type (WT) and the indicated *PTB* misexpression lines for buds (B), cauline leaves (CL), and rosette leaves (RL). M designates marker, and displayed bands correspond to 500, 400, and 300 bp. Black and open circles indicate bands derived from the exon inclusion and skipping variant, respectively.

(B) and **(C)** RT-PCR analysis of *PIF6* splicing in dry seeds from the indicated genotypes. Marker and symbols are as described for **(A)**. RT-PCR products from three independent replicates were quantified using a bioanalyzer **(C)**. Displayed data are mean values ± SD.

(D) Germination assays of wild-type, ami1&2, and OE1 seed in the absence (left panel) or presence of 0.5 µM (middle) or 2 µM (right) ABA in the medium. Data are mean values from three independent experiments and total six replicates; error bars show SE.

(E) Germination rates of the indicated genotypes 57 h after transfer of the seeds to light in the absence or presence of ABA in the medium. Data are mean values from three independent experiments and total six replicates (17 replicates for the wild-type); error bars show SE.

AS Control by At-PTBs

All three At-PTBs have previously been demonstrated to exhibit splicing regulatory potential in an autoregulatory mechanism (Stauffer et al., 2010). Furthermore, the close homologs PTB1 and PTB2 also displayed cross-regulation, while the distantly related PTB3 had no effect on the splicing of the pre-mRNAs of the two other homologs. Our current study further supported the occurrence and specificity of these auto- and cross-regulatory circuits on transcript and protein level (Figure 1) but also suggested development-dependent alterations (see Supplemental Figure 6 online). Interestingly, while a few hundred splicing regulatory targets of PTB1 and PTB2 were identified, only two PTB3-dependent AS events were confirmed. One major difference in comparison of the *PTB1/2* and *PTB3* overexpression lines was only a modest PTB3 increase for the corresponding OE line, compared with the much larger PTB level increase seen in lines OE1 and OE2. However, as our computational analysis for the identification of PTB-dependent AS events was based primarily on splicing ratio changes in lines with diminished PTB levels compared with the wild-type and OE lines, the different overexpression levels are unlikely to explain our finding of a varying extent of AS control by PTBs. Thus, PTB3 might not play a major role in AS control in *Arabidopsis*, but rather have other functions in RNA metabolism. In line with this hypothesis, a pumpkin homolog of At-PTB3 was shown to be part of a phloem-mobile ribonucleoprotein (Ham et al., 2009), the assembly of which requires phosphorylation of the PTB protein (Li et al., 2011).

PTBs have been intensively studied in mammals, where they play an important role during neuronal differentiation (Boutz et al., 2007; Makeyev et al., 2007; Yap et al., 2012). Recent studies indicated that the mode of action of many splicing factors, including PTBs, is highly position dependent (Xue et al., 2009; Llorian et al., 2010). Analyzing inclusion rates of cassette exons, the dominant type of reported AS in mammals, 196 PTB-repressed and 67 PTB-activated instances upon knockdown of PTB in HeLa cells were identified (Llorian et al., 2010). Interestingly, a similar total number of At-PTB1/2–dependent AS events were found in this study, with an overrepresentation of cassette exons compared with all events. In contrast with PTB regulation in mammals, the inclusion of most of these cassette exons was At-PTB1/2–dependent, which is consistent with the

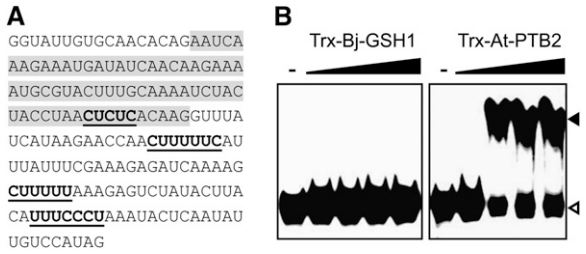

Figure 7. At-PTB2 Binds Directly to an At-*PIF6* RNA Fragment in Vitro.

(A) RNA sequence used for EMSA encompassing the regulated *PIF6* cassette exon (shaded in gray), partial upstream and complete downstream intron, and several pyrimidine-rich stretches (bold and underlined).
(B) EMSA of *PIF6* RNA fragment in the absence of added protein (−), with an unrelated control protein (Trx-Bj-GSH1) or Trx-At-PTB2 fusion protein. Increasing protein concentrations are from left to right. Open and closed arrowheads correspond to free probe and RNA-protein complex, respectively.

previously described auto- and cross-regulation of At-PTB1 and At-PTB2, leading to PTB-dependent inclusion of a poison exon (Stauffer et al., 2010). The opposite mode of autoregulation (i.e., PTB-mediated exon skipping) is found for At-PTB3 (Stauffer et al., 2010) and mammalian PTBs (Wollerton et al., 2004; Spellman et al., 2007). Furthermore, it has been reported that PTB-activated exons typically had PTB binding sites downstream of the exon, while PTB-repressed exons contained pyrimidine motifs upstream of or within the cassette exon (Llorian et al., 2010). Future work needs to address whether there is a similar correlation between binding positions and splicing activities of At-PTBs. Probing of RNA–protein interactions will also reveal if all of the identified targets are directly regulated by At-PTBs, as suggested by the reciprocal AS ratio changes upon up- and downregulation of these splicing factors, or if some alterations are mediated by other, PTB-controlled splicing regulators. In the case of *PIF6*, a direct, in vitro interaction between an RNA probe containing the alternatively spliced region and PTB2 protein was demonstrated (Figure 7).

Besides its well-established role in the regulation of cassette exons, mammalian PTB recently was found to repress splicing of introns within pre-mRNAs of neuron-specific genes (Yap et al., 2012). The resulting mRNAs are recognized as incompletely spliced, leading to their nuclear degradation. We show that At-PTB1 and -PTB2 stimulate and repress intron splicing, as well as alter 5′ splice site choice. However, only few At-PTB1/2–regulated alt 3′ events were identified, suggesting preferential regulation of certain AS types by At-PTBs.

Analysis of plant lines expressing single amiRNAs targeting either At-*PTB1* or -*PTB2* revealed both redundant and specific splicing regulatory functions of the two homologs (Figure 5). In mammals, distinct splicing regulatory activities of PTB and its neuronal homolog nPTB have been reported as an important means for altering gene expression in the course of neuronal differentiation (Boutz et al., 2007; Makeyev et al., 2007). Thus, it will be interesting to see whether relative levels or activities of At-PTB1 and -PTB2 also change in response to certain signals, thereby inducing altered splicing programs.

At-PTB–Mediated *PIF6* Splicing Regulates Seed Germination

The *PIF6* locus generates two splicing variants, encoding predicted proteins either containing or lacking a protein domain due to inclusion or skipping of the corresponding cassette exon. *PIF6* previously has been shown to be expressed during seed development and to have important implications in seed germination (Penfield et al., 2010). Overexpression of the shorter transcript variant, predicted to generate a protein without the DNA binding domain, reduced primary seed dormancy, while no such effect was found for the longer version. Interestingly, we could show that AS of *PIF6* is regulated by PTB1 and PTB2. Misexpression of these splicing regulators perturbed *PIF6* splicing, with increased and reduced levels of exon skipping upon down- and upregulation of PTB1/2, respectively. In line with previous findings (Penfield et al., 2010), a shift toward the shorter variant accelerated seed germination. Furthermore, we observed delayed germination upon change of the splicing ratio into the opposite direction. The seed germination

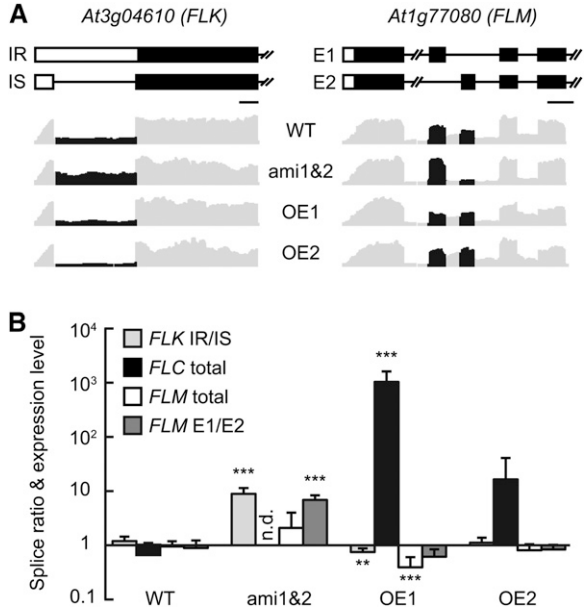

Figure 8. Changes in Splicing Pattern and Expression of the Flowering Regulator Genes *FLK*, *FLC*, and *FLM* in Lines with Altered PTB Levels.

(A) Partial gene models and corresponding coverage plots derived from RNA-seq data for the alternatively spliced regions in the 5′ UTR of the *FLK* (left) and the cds of the *FLM* (right) pre-mRNAs. Details of cartoon depiction as described in legend to Figure 3. WT, the wild-type. IR, intron retention; IS, intron splicing.
(B) Ratio of IR to IS variant of *FLK* mRNA, total levels of *FLC* and *FLM* mRNAs, and ratio of *FLM* splicing isoforms including the first (E1) or second (E2) of the mutually exclusive exons. Values were determined by reverse transcription and quantitative PCR of the indicated genotypes from plants at rosette stage. In the ami1&2 lines, *FLC* mRNA amount was reduced to an undetectable level (n.d.). Data are mean values with SD (n = 5 to 9, each sample derived from three individuals). Highly significant changes compared with corresponding wild-type samples are indicated (***$P < 0.0001$; **$P < 0.0003$ according to Student's t test).

phenotype of the *PTB* misexpression lines depended on the presence of exogenous ABA, and additional links between splicing factors and ABA signaling have recently been reported (Duque, 2011). For example, mutants in the splicing regulator SR45 showed ABA hypersensitivity (Carvalho et al., 2010), and the ABA signaling component *ABSCISIC ACID INSENSITIVE3* was found to be subjected to developmentally controlled AS, with one splicing variant accumulating at the end of seed maturation (Sugliani et al., 2010). Interestingly, *PIF6* showed organ-specific splicing patterns when comparing leaves, flowers, and dry seeds, and these differences were weakened or abrogated in the *PTB* misexpression lines. This organ-specific splicing switch might be due to changes in the expression or activity of PTB1 and PTB2 as well as the involvement of additional splicing factors.

At-*PTB* Misexpression Reveals Intricate Links between AS and Expression for Flowering Regulators

Control of flowering time is crucial for the reproductive success of plants, and numerous previous studies have provided evidence that RNA processing factors play key roles in these complex regulatory networks (Terzi and Simpson, 2008). Accordingly, several links between AS and timing of flowering have been established. The circadian clock component At-GRP7, which autoregulates its own expression via AS, has been described as a novel factor of the autonomous pathway (Streitner et al., 2008) and controls flowering time by repressing the floral repressor FLC (Michaels and Amasino, 1999; Sheldon et al., 1999; Lee et al., 2000). Similarly, FLK also acts as a floral activator by repressing FLC (Lim et al., 2004; Mockler et al., 2004), and recent characterization of the Arg dimethyltransferase *atprmt5* mutant showed that FLK levels, and consequently *FLC* expression, depend on AS of the *FLK* pre-mRNA. While elevated levels of retention of the first intron within *FLK* were found to correlate with *FLC* induction (Deng et al., 2010), the opposite direction of change was found in the *PTB1/2* misexpression lines, suggesting a more complex regulatory relation between *FLK* and *FLC*. The same AS event within *FLK* pre-mRNA was also found to be affected in plants carrying mutations in SERRATE and cap binding complex proteins (Laubinger et al., 2008), further highlighting the multifactorial control of this AS event.

Evidence for additional connections between AS and flowering time control was provided in a study analyzing flowering induction in response to elevated temperature (Balasubramanian et al., 2006). The authors identified *FLM*, a member of the *FLC*-like clade that also represses flowering (Ratcliffe et al., 2001), as a major quantitative trait locus modulating thermal induction of flowering. Furthermore, thermal induction affected the expression of several RNA processing factors, including SR proteins, and led to an altered AS pattern of *FLM*. It was therefore speculated that a shift in AS of *FLM* might provide a mechanism to overcome the repressive effect of FLM in response to elevated temperature, assuming that the splicing variants might have different functional implications. In support of this hypothesis, sequence analysis of the protein isoforms predicted to be produced from the *FLM* splicing variants with mutually exclusive exons revealed the occurrence of distinct interaction motifs (Severing et al., 2012). Interestingly, we also found an alteration of *FLM* splicing upon *PTB1/2* misexpression, with an increase of the splicing variant formerly described as a floral repressor (Ratcliffe et al., 2001) upon simultaneous knockdown of the two close At-PTB homologs. In summary, altered levels of At-PTB1 and -PTB2 resulted in pronounced changes of several key flowering regulators. Knockdown of PTB1/2 caused down- and upregulation of the floral repressors *FLC* and *FLM*, respectively, while AS of the floral inducer *FLK* showed a shift toward an intron retention variant that had previously been demonstrated to be nonfunctional (Deng et al., 2010). We did not find any evidence for alternative processing of *FLC* mRNAs or changes of the corresponding *COOLAIR* antisense transcripts that might counteract the massive alterations in the *FLC* sense transcript levels. Intriguingly, in contrast with the previously described mutants with altered splicing patterns for flowering regulators, the *PTB* misexpression lines flowered at the same time as the wild-type, suggesting compensation of the opposing changes of floral repressors and activators. Our findings thereby further highlight the tight interconnections between these flowering regulators and suggest that AS plays an important role in modulating their expression. Given the observation of simultaneous, compensating changes of AS for several flowering regulators upon alteration of PTB levels, it can be speculated that additional factors are required to specifically change single AS events and thereby affect the onset of flowering. Likely candidate factors are SR proteins, which are known to antagonize hnRNP protein functions in animals. In conclusion, having identified a large and diverse collection of PTB-regulated AS events, future work needs to further elucidate the underlying regulatory mechanism and define complete sets of both *cis*- and *trans*-acting factors. These studies will pave the way for an understanding of the plant splicing code, representing the molecular basis of the highly complex AS patterns linked to manifold biological processes in plants.

METHODS

Plant Growth and Material

For sterile growth, *Arabidopsis thaliana* seeds were surface sterilized with 3.75% NaOCl and 0.01% Triton X-100 and plated on half-strength Murashige and Skoog medium (Duchefa) containing 2% Suc and 0.8% phytoagar (Duchefa). Following stratification for 2 d in darkness at 4°C, plates were transferred to continuous light (~20°C, ~60% humidity). After cultivation for 10 to 12 d, seedlings were sampled or transferred to soil and further grown under long-day growth conditions (16 h light, 8 h darkness, ~20°C, ~60% humidity). *Arabidopsis* was stably transformed using a previously described floral dip method (Clough and Bent, 1998). For selection, seeds were sown on soil followed by spraying of 2- to 3-week-old plants with 0.1% Basta (Bayer) or grown under sterile conditions on medium containing 50 μg/mL kanamycin for plant selection and 200 μg/mL Cefotaxime to prevent bacterial growth. Seeds of following generations were grown under sterile conditions on medium containing 25 μM Basta or 20 μg/mL kanamycin. Single T-DNA insertion lines for At-*PTB1* (*atptb1-1*, SALK_013673C), At-*PTB2* (*atptb2-1*, SAIL_736_B12), and At-*PTB3* (*atptb3-1*, GK-078G10) were obtained from the Nottingham Arabidopsis Stock Centre.

Cloning Procedures

amiRNA constructs to downregulate At-PTB1/2/1&2/3 expression were designed using the WMD3 Web tool (http://wmd3.weigelworld.org;

Ossowski et al., 2008) and cloned using the primers given in the oligonucleotide list (see Supplemental Table 4 online) as described in the provided protocol (http://wmd3.weigelworld.org/downloads/Cloning_of_artificial_microRNAs.pdf). Thereafter, the resulting amiRNA precursors plus flanking vector sequences were recombined into pDONR201 (Invitrogen) via Gateway cloning (Invitrogen). Subsequently, the entry clones were sequenced and recombined into the plant transformation vector pB7WG2 (Karimi et al., 2002).

To clone overexpression constructs of At-*PTB1/2/3*, the respective coding regions were PCR amplified from seedling cDNA using the primers given in Supplemental Table 4 online. The amplification products were digested with *Bam*HI-*Sal*I and cloned into the accordingly digested vector pBinAR (Höfgen and Willmitzer, 1992). For expression of the *PIF6* splicing variants under control of the cauliflower mosaic virus 35S promoter, cds of the four possible splicing variants were cloned into pBinAR via *Bam*HI-*Sal*I. Splicing variants *At3g62090.1* (cassette exon inclusion) and *At3g62090.3* (cassette exon skipping) contain an intron within the 5′ UTR and therefore have a start codon at a later position compared with *At3g62090.2* (cassette exon inclusion). A fourth variant, here named *At3g62090.2/3*, with an early start codon and cassette exon skipping could also be detected. The cds of *At3g62090.1*, *At3g62090.2*, *At3g62090.2/3*, and *At3g62090.3* were amplified with oligos DNA43/44, DNA45/44, DNA45/44, and DNA43/44, respectively, followed by an additional PCR with DNA46 and the listed reverse primers for addition of a complete Flag tag.

To generate antibodies directed against *Arabidopsis* PTB proteins, the coding regions of At-*PTB1*, -*PTB2*, and -*PTB3* were PCR amplified from seedling cDNA using the primers indicated in the oligonucleotide list. The amplification products for *PTB1* and *PTB2* were digested with *Pci*I-*Not*I, whereas the *PTB3* product was digested with *Nco*I-*Not*I. Subsequently, the At-*PTB*–specific inserts were cloned in the *Nco*I-*Not*I–digested expression vector pETM-20 (Hothorn et al., 2003), resulting in constructs encoding Thioredoxin A-His6-tagged PTB proteins with a TEV protease cleavage site. For generation of a PIF6-specific antibody, the cds of the transcript variant *At3g62090.3* was amplified with primers DNA55 and DNA56. The resulting PCR product was digested with *Nco*I-*Kpn*I (using partial digestion as *Nco*I also cuts within the *PIF6* sequence) and cloned into the corresponding sites of pETM-20.

Transient Expression of PIF6 Constructs in *Nicotiana benthamiana*

Transient construct expression was based on a previously described *Agrobacterium tumefaciens*–mediated leaf infiltration assay (Wachter et al., 2007) and included the gene silencing suppressor P19. Samples for protein extraction were taken 5 d after infiltration.

Purification of Recombinant Proteins, Antibody Generation, and Immunoblot Analyses

Recombinant proteins were expressed in the *Escherichia coli* strain *Rosetta gami* DE3 at 16°C overnight, after induction with the following final concentrations of isopropyl-β-D-thiogalactopyranoside: At-PTB1, 0.1 mM; At-PTB2, 0.1 mM; At-PTB3, 0.5 mM; and At-PIF6, 0.1 mM. Bj-GSH1 (Hothorn et al., 2006) was expressed at 28°C overnight after induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside. Protein purification was performed using Ni-TED resin (Macherey-Nagel) according to the manufacturer's instructions. In brief, cell lysis was performed using a French pressure cell (Aminco; 3×, 1000 p.s.i.), and lysates were treated with 50 μg/mL DNase for 15 min at room temperature and subsequently cleared by centrifugation for 30 min at 12,000 rpm and 4°C. For native purification, tagged At-PTB or Bj-GSH1 proteins were bound to Protino Ni-TED resin for 45 min at room temperature. After several washing steps, proteins were eluted first three times with 150 mM imidazole, then three times with 200 mM imidazole. Finally, elution fractions were combined, buffer exchanged to 50 mM NaH_2PO_4 and 200 mM NaCl, pH 8.0, using Zeba desalt spin columns (Pierce), and concentrated (Pierce concentrators, 20 mL/20K) to a final concentration of ~1 mg/mL. Recombinant At-PTB1 and At-PTB2 proteins were directly used for immunization of rabbits, whereas the recombinant At-PTB3 protein was TEV cleaved, followed by removal of the tag by a second column purification step prior immunization. The recombinant PIF6 fusion protein was purified under denaturing conditions. The pellet from the centrifugation after DNase treatment was washed once, followed by resuspension in 50 mM NaH_2PO_4, 300 mM NaCl, and 8 M urea, pH 8.0. After incubation for 1 h at 4°C and centrifugation (20 min at 12,000 rpm and 4°C), the supernatant was incubated with Protino Ni-TED resin for 1 h at room temperature. After several washing steps, proteins were eluted first three times with 200 mM imidazole, then three times with 300 mM imidazole in 8 M urea–containing buffer. Several dialysis steps with decreasing urea concentrations were performed (6, 4, 2, and 0.5 M urea in 50 mM NaH_2PO_4 and 200 mM NaCl, pH 8.0), followed by protein concentration as described above.

Rabbits were immunized at least five times with ~0.1 mg recombinant protein (BioGenes). Antibodies were purified from raw sera by affinity purification using recombinant protein blotted on nitrocellulose membranes. Therefore, raw sera were diluted 1:5 in 2% skim milk in Tris buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.5), and binding to the antigen was performed at 4°C for 1 h. Subsequently, nitrocellulose stripes were washed three times for 10 min with 2% skim milk-TBS, followed by short washing steps with TBS and water. Antibodies were eluted in two steps by a pH shift. Therefore, stripes were incubated for 3 min in elution buffer 1 (5 mM Gly and 0.5 M NaCl, pH 2.8), and elution was performed on ice with robust shaking. After the first elution, the supernatant was neutralized immediately with Tris, pH 8.0, to a final concentration of 90 mM Tris. The elution was repeated with elution buffer 2 (5 mM Gly and 0.5 M NaCl, pH 2.2), and elution fractions were combined and supplemented with 0.1% BSA. Finally, all elution fractions were concentrated and antibody titers as well as specificities were determined by immunoblotting.

For immunoblot analyses, proteins were extracted from indicated genotypes and tissues using 300 μL extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% [v/v] Tween 20, and 0.1% [v/v] β-mercaptoethanol). SDS-PAGE and immunoblots were conducted according to standard protocols using the indicated amounts of total protein, followed by chemiluminescence detection (Super Signal West Dura; Pierce) according to the manufacturer's instructions. For an estimation of protein quantities in the At-PTB overexpression lines, immunoblot signals were quantified using ImageJ (Schneider et al., 2012).

EMSAs

The *PIF6* probe was obtained by in vitro transcription from a PCR-generated DNA template (oligos DNA140/141), followed by 5′ ^{32}P-labeling and gel purification as described before (Wachter et al., 2007). For renaturation, RNA was incubated in 1× binding buffer (20 mM HEPES, 0.2 mM EDTA, 100 mM KCl, 3.125 mM $MgCl_2$, and 20% glycerol [w/v], pH 7.9) for 3 min at 55°C, followed by 5 min at room temperature. Recombinant proteins Trx-At-PTB2 and Trx-Bj-GSH1 (Hothorn et al., 2006) were dialyzed against 1× binding buffer and treated with RNase inhibitor (RiboLock; Fermentas). EMSA reactions of 10 μL total volume contained 10 fmol RNA, 0.1 μg/μL BSA, 3.75 ng/μL tRNA, and varying amounts of purified, recombinant protein (~70 to 200 ng) in 1× binding buffer and were incubated for 10 min at 25°C, followed by native electrophoresis [6% polyacrylamide gel in 0.5× Tris-borate buffer (45 mM Tris, 45 mM borate, 0.5 mM EDTA, pH 8.0) and 5% glycerol] at 4°C for ~3 h and 10 V/cm. Gels were dried and visualized using phosphor imaging (GE Healthcare).

RNA Isolation and RT-PCR Analyses

Total RNA was extracted from ~100 mg of plant tissue using the Universal RNA purification kit (EURx), including an on-column DNaseI treatment

performed according to the manufacturer's instructions. RNA from 75 to 100 mg dry seeds was isolated using a borate extraction protocol as described previously (Penfield et al., 2005). Reverse transcription of total RNA was performed with dT_{20} using AMV reverse transcriptase native (EURx) following the supplied protocol. RT-PCR products were separated and visualized using 2% agarose Tris-acetate (TAE, 40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8.0) gels or 12% native polyacrylamide gels (Tris-Gly native running buffer) and ethidium bromide staining. For quantification of RT-PCR products, DNA1000 chips on an Agilent 2100 bioanalyzer were used according to the manufacturer's instructions. RT-PCR products were cloned into pGEM-T vector (Promega) before sequencing (Eurofins). Quantitative PCR of cDNA samples was performed using the Bio-Rad CFX384 real-time PCR system and MESA GREEN qPCR Mastermix Plus (Eurogentec) as described previously (Stauffer et al., 2010).

Preparation of TruSeq mRNA Libraries and Illumina Sequencing

Libraries were prepared using the Illumina TruSeq sample prep kit (Illumina) according to the manufacturer's instructions (TruSeq RNA Sample Preparation v2 Guide, version November 2010). Starting materials were 4 to 4.5 μg of total RNA, isolated from 11-day-old seedlings, as described above. For the adapter ligation step, the RNA Adapter Indexes 10 (AR010) and 13 (AR013) were used. Following the PCR enrichment step using half the sample volume, libraries were subjected to an additional gel purification step. A size range of 250 to 350 bp was extracted from a 2% agarose gel and purified using the MinElute gel extraction kit (Qiagen). Cluster generation was performed on a Cluster Station (Illumina) or cBot (Illumina) according to the manufacturer's instructions. Sequencing was performed with one sample per lane, and 8 to 10 pM solutions of denatured libraries were used to generate ~800,000 raw clusters per mm^2 on the flow-cell surface. Sequencing was performed according to the manufacturer's protocols on a Genome Analyzer GAIIx (Illumina) using the TruSeq SBS Sequencing Kit v5 (36 cycle; Illumina). Sequencing control software was SCS version 2.8 and RTA1.8.7. Sequencing runs were performed at 80-bp cycles or 100-bp cycles. For further details, see Supplemental Methods 1 online.

RNA-seq Data Analysis

The RNA-seq read data were aligned to the *Arabidopsis* reference genome (TAIR10) using the spliced alignment tool PALMapper (Jean et al., 2010). Further postprocessing to achieve an optimal filter setting and to resolve multimappers to unique locations was done using the RNA-geeq toolbox (http://bioweb.me/rnageeq). The TAIR10 annotation was transformed into a splicing graph representation and was augmented with additional splice evidence from alignment data using Spladder. Canonical AS events were extracted from the generated splicing graph and transformed into genelets (a minimal gene with two transcript isoforms). Differential testing of alternative splice isoform expression and differential gene expression estimation based on negative binomial tests taking biological variance into account were made with rDiff (Stegle et al., 2010; http://bioweb.me/rDiff) and DESeq (Anders and Huber, 2010), respectively, on the aforementioned genelets. The resulting ranked lists for different conditions were combined in a spreadsheet program. For NMD feature evaluation, an analysis pipeline was implemented in MATLAB, Python, and as shell scripts. For further details, see Supplemental Methods 1 online.

Functional Clustering of At-PTB1/2–Regulated Genes

Functional clustering was performed using the MapMan software (http://mapman.gabipd.org; Thimm et al., 2004). All analyzed genes were assigned a gene function based on the corresponding GO terms listed in TAIR10. Based on these GO terms, genes were clustered in 35 different functional groups and further combined as depicted in Supplemental Data Set 3 online.

Germination Assays

Seeds from 10 plants of each genotype simultaneously grown under greenhouse conditions were harvested at the same time and after-ripened at 4°C for at least 2 months. For each experiment, 80 to 100 seeds of each genotype were surface sterilized for 5 min and sown on half-strength Murashige and Skoog medium (as described above) containing ABA (Sigma-Aldrich) in the indicated concentrations. ABA was dissolved in 100% methanol, and a volume of methanol corresponding to the highest ABA concentration was added to the control medium. After stratification for 2 d in darkness at 4°C, plates were transferred to a long-day growth chamber (~20°C, ~60% humidity, 16 h light). Seeds were scored as germinated at the stage of radicle emergence.

Flowering Time Determination

Seeds of each genotype were sown on soil and transferred to short-day (8 h light) or long-day (16 h light) conditions after stratification for 2 d in darkness at 4°C. For each genotype and growth condition, nine to 20 plants were transferred to single pots 2 weeks after transfer to light and arranged randomly to avoid positional variations of growth conditions. Plants were scored as flowering once inflorescence stems reached a length of 1 cm. Flowering time was determined by counting rosette leaf numbers or days after transfer to light, for long- and short-day conditions, respectively.

Accession Numbers

Sequence data for genes in this article can be found in The Arabidopsis Information Resource databases under the following accession numbers: *PTB1* (*At3g01150*), *PTB2* (*At5g53180*), *PTB3* (*At1g43190*), *PIF6* (*At3g62090*), *FLK* (*At3g04610*), *FLC* (*At5g10140*), and *FLM* (*At1g77080*). Accession numbers of further analyzed genes are given in Figures 3 and 4 and Supplemental Figures 8, 9, 11, and 12 online. Supplemental Data Sets 1 to 3 online are available at datadryad.org (doi:10.5061/dryad.6k1p6). RNA-seq data have been deposited in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE41433.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Altered At-*PTB* Transcript Levels in amiRNA and Overexpression Lines of T0 Generation.

Supplemental Figure 2. Specificities of Purified At-PTB Antibodies.

Supplemental Figure 3. Exposure Time-Dependent At-PTB1 Immunosignals.

Supplemental Figure 4. Genotyping and Immunoblot Analyses of At-*PTB* T-DNA Insertion Lines.

Supplemental Figure 5. Mapping of T-DNA Positions in At-*PTB1*, -*PTB2*, and -*PTB3* Insertion Lines.

Supplemental Figure 6. PTB2 and PTB3 Protein Levels in *PTB* Misexpression Lines at the Rosette Stage.

Supplemental Figure 7. RNA-seq–Based *PTB* Expression Analysis in the Corresponding Misexpression Lines.

Supplemental Figure 8. Validation of Putative PTB3 Splicing Regulation Targets.

Supplemental Figure 9. Partial Gene Models and Corresponding Coverage Plots for Described Cassette Exon Events.

Supplemental Figure 10. Sequences of Main RT-PCR Products Corresponding to Described Splicing Variants.

Supplemental Figure 11. Partial Gene Models and Corresponding Coverage Plots for Selected Intron Retention and Alternative 5′ Splice Site Events.

Supplemental Figure 12. Validation of PTB1/2 Functioning in Alternative 3′ Splice Site Choice.

Supplemental Figure 13. Visualization of RT-PCR Products for Alternative Splicing Events in Single amiRNA and *PTB3* Misexpression Lines.

Supplemental Figure 14. Germination Assays of Wild-Type and *PTB* Misexpression Lines and AS Products of *PIF6*.

Supplemental Figure 15. Flowering Time Analysis of *PTB* Misexpression Lines under Long- and Short-Day Conditions.

Supplemental Figure 16. Analysis of *FLC* Antisense Expression in Wild-Type and *PTB* Misexpression Lines.

Supplemental Table 1. Overview of Utilized amiRNAs and Their Targets.

Supplemental Table 2. Read Statistics of RNA-seq Data.

Supplemental Table 3. Frequencies of AS Event Positions in the mRNA and NMD Features.

Supplemental Table 4. List of Oligonucleotides.

Supplemental Methods 1. Sequencing of mRNA Libraries and Computational Methods.

Supplemental Data Set 1. Computational Analysis of Transcriptome-Wide AS Patterns in *PTB1/2* Misexpression Lines.

Supplemental Data Set 2. Computational Analysis of Transcriptome-Wide AS Patterns in *PTB3* Misexpression Lines.

Supplemental Data Set 3. Detailed List of GO Terms.

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AUTHOR CONTRIBUTIONS

C.R., E.S., G.R., and A.W. designed research. C.R., E.S., A.K., G.W., G.D., G.R., and A.W. performed research. All authors contributed to data analysis and discussion. A.W., C.R., and E.S. wrote the article.

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5.2. Review article

5.2.1. A. Wachter, C. Rühl, **E. Stauffer**, (**2012**). *The role of polypyrimidine tract-binding proteins and other hnRNP proteins in plant splicing regulation.* Frontiers in Plant Science 3:81.

Personal contributions

In this review article, I drafted the section describing the mechanisms of hnRNP splicing regulation and constructed figure 1. Together with A. Wachter and C. Rühl, the manuscript was prepared and discussed.

5.2.1. A. Wachter, C. Rühl, **E. Stauffer**, (2012). *The role of polypyrimidine tract-binding proteins and other hnRNP proteins in plant splicing regulation.* Frontiers in Plant Science 3:81.

the initial step in interchromosomal homologous recombination, thus indicating that GTBP1 is a negative regulator of telomere length, being essential for both structure and function of tobacco telomeres.

Interestingly, hnRNP protein homologs are also found in plant organelles. Chloroplastidic ribonucleoproteins (cpRNPs) have been described to play a role in chloroplast RNA processing steps (Tillich et al., 2010), such as 3′ end processing (Schuster and Gruissem, 1991) and RNA editing (Hirose and Sugiura, 2001), as well as in regulating transcript stability (Nakamura et al., 2001). For example, knockout of the cpRNP C31A gave rise to alterations in both editing and steady state levels of distinct sets of chloroplast mRNAs (Tillich et al., 2009). cpRNPs possess twin RRMs and are subject to light-dependent phosphorylation (Tillich et al., 2010), which has been demonstrated to reduce the RNA *in vitro* binding affinity in case of CP28 (Lisitsky and Schuster, 1995). Furthermore, analysis of mutants suggests that cpRNPs have both specific and combinatorial functions, as it is also observed for many hnRNPs.

CONCLUSION

Global analyses of transcript data have revealed the widespread occurrence of AS in plants, bearing an enormous potential for a functional expansion of the transcriptome. Although at this point it remains unclear which fraction of all AS events plays a role in plant gene expression, a steadily growing list of studies provides compelling evidence for important implications of AS in numerous fundamental biological processes. Thus, one of the most intriguing questions in current AS research in plants is how AS can be regulated and how it can be coordinated with other processes to trigger specific splicing programs in response to internal and external cues. Important lessons can be learned from splicing research performed in animal systems, however, previous studies also have highlighted numerous distinct features of splicing in plants and animals. Recent progress in the characterization of SR and hnRNP proteins from plants represents an important step toward deciphering the plant splicing code. It can be anticipated that the combinatorial action of those splicing factors play a pivotal role in determining the complex AS patterns observed in plants, and thereby critically contributes to the regulation of gene expression in the context of diverse intrinsic processes as well as in response to external signals.

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REVIEW ARTICLE
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The role of polypyrimidine tract-binding proteins and other hnRNP proteins in plant splicing regulation

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Alternative precursor mRNA splicing is a widespread phenomenon in multicellular eukaryotes and represents a major means for functional expansion of the transcriptome. While several recent studies have revealed an important link between splicing regulation and fundamental biological processes in plants, many important aspects, such as the underlying splicing regulatory mechanisms, are so far not well understood. Splicing decisions are in general based on a splicing code that is determined by the dynamic interplay of splicing–controlling factors and *cis*-regulatory elements. Several members of the group of heterogeneous nuclear ribonucleoprotein (hnRNP) proteins are well known regulators of splicing in animals and the comparatively few reports on some of their plant homologs revealed similar functions. This also applies to polypyrimidine tract-binding proteins, a thoroughly investigated class of hnRNP proteins with splicing regulatory functions in both animals and plants. Further examples from plants are auto- and cross-regulatory splicing circuits of glycine-rich RNA binding proteins and splicing enhancement by oligouridylate binding proteins. Besides their role in defining splice site choice, hnRNP proteins are also involved in multiple other steps of nucleic acid metabolism, highlighting the functional versatility of this group of proteins in higher eukaryotes.

Keywords: alternative splicing, splicing regulation, polypyrimidine tract-binding protein, PTB, heterogeneous nuclear ribonucleoprotein, hnRNP

INTRODUCTION

The majority of plant genes contain intronic regions, which need to be removed with a high degree of precision and efficiency in the process of precursor messenger RNA (pre-mRNA) splicing. In the recent years, the tremendous increase in plant transcript data has revealed that a significant proportion of all genes generates transcript variants due to alternative splicing (AS) of pre-mRNAs (Wang and Brendel, 2006; Filichkin et al., 2010; Zhang et al., 2010). This enormous expansion of the transcriptome has major implications for both proteome diversity and gene regulation via altered mRNA features, such as the mRNA turnover rate, and essential functions have been ascribed to splicing variants in the context of manifold biological processes in plants (Reddy, 2007).

Numerous AS events have been demonstrated to be regulated in a spatial–temporal manner or by exogenous stimuli, requiring complex control mechanisms for correct splice site choice. While regulation of AS has been intensively studied in animals (Chen and Manley, 2009), only few reports are available that describe *cis*-regulatory sequences and *trans*-acting factors controlling AS in plants (Reddy, 2007; Barbazuk et al., 2008; Wachter, 2010). Two major classes of universal splicing regulatory factors are constituted by the serine/arginine-rich (SR) proteins and members of the heterogeneous nuclear ribonucleoprotein (hnRNP) protein family, which have been reported to act antagonistically in several AS events. For example, in case of the *Dscam* exon 6 cluster, the specific interplay between the hnRNP protein HRP36 and an SR protein is required for correct inclusion of a single cassette exon (Olson et al., 2007). Whereas earlier studies had indicated that hnRNP and SR proteins typically act as repressors and activators of splicing, respectively, it is now becoming evident that the mode of action of splicing regulatory factors often is context-dependent. On one hand, the effect of splicing factors can vary dependent on their binding position within the pre-mRNA. On the other hand, the splicing outcome is often established by the interaction of multiple components of the splicing machinery and additional regulatory factors.

The original definition of hnRNP proteins was based on their experimental identification as major protein constituents of high molecular weight RNA complexes. Subsequently, further proteins with similar characteristics or homology to a previously described hnRNP protein were added to this list, which nowadays comprises a diverse group of proteins that do not share one certain structural or functional feature (Martinez-Contreras et al., 2007; Han et al., 2010). However, given their ability to associate with RNA and single-stranded (ss) DNA, all hnRNP proteins contain RNA recognition motifs (RRMs) or other functionally equivalent domains, including KH domains, quasi-RRMs, and additional types of atypical RRMs (Han et al., 2010). Due to the presence of those domains, most hnRNP proteins can bind to a broad spectrum of ss nucleic acid, however, more stringent experimental conditions allowed the definition of specific binding sequences (Martinez-Contreras et al., 2007; Han et al., 2010). Furthermore, numerous cellular parameters such as the physico-chemical environment, local protein concentration, and interaction with additional factors can critically alter the binding characteristics of hnRNP proteins, thereby providing additional layers of their regulatory potential.

Interestingly, a recent study by Mackereth et al. (2011) has revealed that the U2 auxiliary splicing factor (U2AF) 65 undergoes multi-domain conformational selection, dependent on the strength of the bound polypyrimidine tract. According to their model, with increasing strength of the polypyrimidine tract, a larger fraction of U2AF65 proteins is captured in their open conformation, which represents the domain arrangement that efficiently triggers spliceosomal assembly. A similar mechanism might also be found for some hnRNP proteins, given their nature as multi-domain proteins and their ability to bind diverse sequence motifs. Besides nucleic acid binding domains, most hnRNP proteins contain additional domains linked to their multifaceted functions in nucleic acid metabolism, including chromatin remodeling, pre-mRNA splicing, control of mRNA stability and modifications, and mRNA transport (Martinez-Contreras et al., 2007; Han et al., 2010). This complexity is further increased by the occurrence of both AS of many hnRNP pre-mRNAs and different types of post-translational protein modifications with important implications for hnRNP functions (Martinez-Contreras et al., 2007; Han et al., 2010).

Most of the currently available information on hnRNP proteins is based on studies in the mammalian system. However, homology-based searches for related proteins from plants (Wang and Brendel, 2004) and first reports on the functional characterization of some of them have highlighted the presence and important roles of hnRNP proteins in plants. In this review, we will discuss the potential of hnRNP proteins in plant splicing regulation. First, a brief insight into the most prevalent splicing regulatory mechanisms of hnRNP proteins will be given. Most of these studies have been performed in human or animal systems, however, it seems likely that similar mechanisms are also used by plant hnRNP proteins. Second, the current state of the art on the functions and biological implications of polypyrimidine tract-binding (PTBs) proteins, an example of extensively studied hnRNP proteins with splicing regulatory functions in both animals and plants, will be reviewed. Third, an overview of the experimental reports on other plant hnRNP proteins, with a focus on their role in the regulation of splicing, will be presented. In summary, this review will highlight the recent progress in studying the role of hnRNP proteins in plant splicing regulation. Further, by comparison with the mammalian system, it will be pointed out that hnRNP proteins might play an even more widespread role in controlling splicing decisions in plants, than reflected by the currently available data.

MECHANISMS OF hnRNP PROTEIN-MEDIATED SPLICING REGULATION

Several types of hnRNP proteins are well known regulators of AS and some of them have also been reported to play a role in constitutive splicing. The depletion of hnRNP F (Gamberi et al., 1997) or hnRNP Q (Mourelatos et al., 2001), for instance, resulted in diminished *in vitro* splicing activity. Furthermore, using mass spectrometry, hnRNP proteins were identified as components of purified ribosomes in mammals (Neubauer et al., 1998). A role of hnRNP proteins in generic splicing is also supported by the finding that introduction of intronic binding sites for hnRNPs A/B and F/H triggered splicing *in vitro* and *in vivo*, and that computational prediction revealed an overrepresentation of the corresponding binding sites at both ends of human introns (Martinez-Contreras et al., 2006). Based on these discoveries, it was suggested that some hnRNP proteins might play a more general role in the recognition of splice sites that could be brought together by the interaction of hnRNP proteins bound to flanking intronic regions.

The regulatory outcome of splicing factor binding can be highly position-dependent, as was revealed by several recent studies. Transcriptome-wide maps of RNA binding sites for the neuron-specific splicing factor Nova, for instance, revealed that binding around the alternative 5′ or constitutive 3′ splice site typically induces exon inclusion, whereas exon skipping is often associated with binding sites around the constitutive 5′ splice site or the alternative exon (Ule et al., 2006; Licatalosi et al., 2008). Binding position-dependent splicing regulatory functions have also been described for FOX proteins (Zhang et al., 2008; Venables et al., 2009; Yeo et al., 2009), Mbnl1 (Du et al., 2010), and PTB (Xue et al., 2009; Llorian et al., 2010). Furthermore, in the case of mutually exclusive splicing, where only one of two or more alternative exons is included, hnRNP proteins can induce skipping of one exon and stimulate inclusion of another one. This has been reported for the human pyruvate kinase pre-mRNA, the mutually exclusive splicing of which is regulated by hnRNPs A1 and A2, and PTB (David et al., 2010). It is also more frequently observed that two or more types of hnRNP proteins act in a combinatorial manner, further expanding the repertoire of hnRNP protein-mediated modes of action and functions.

Precursor messenger RNA splicing is confined to the nuclear compartment, in which some hnRNP proteins are highly abundant. However, many hnRNP proteins display nucleo-cytoplasmic shuttling that has been demonstrated to be regulated by post-translational modifications. Shuttling between those compartments might represent a means to limit their splicing regulatory activity in the nucleus. Furthermore, transport of hnRNPs out of the nucleus might be a prerequisite for the cytosolic functions, ascribed to some hnRNP proteins. For example, mammalian PTB was shown to be specifically phosphorylated by the 3′,5′-cAMP-dependent protein kinase, which is required for export of the protein into the cytosol (Xie et al., 2003). In addition to protein phosphorylation, the methylation of arginine residues (Chang et al., 2011) has also been described as a type of post-translational modification with implications for the subcellular distribution of hnRNP proteins. It can be concluded that the splicing regulatory activity of hnRNP proteins can be subject to tight control, and, furthermore, that hnRNP proteins typically act together with other splicing factors to alter splicing decisions. Numerous models for mechanisms of AS control by hnRNP proteins have been suggested and, based on recent findings, it seems likely that they are not mutually exclusive, but rather reflect the versatility of splicing regulation. In the following paragraphs, the most commonly proposed modes of action will be briefly discussed.

hnRNP-MEDIATED DISPLACEMENT OR RECRUITMENT OF SPLICING FACTORS

The most straightforward mode of action is based on a competition between hnRNP proteins and other splicing factors for binding to *cis*-elements having a splicing regulatory function (**Figure 1A**). Negative regulation can be achieved by occlusion

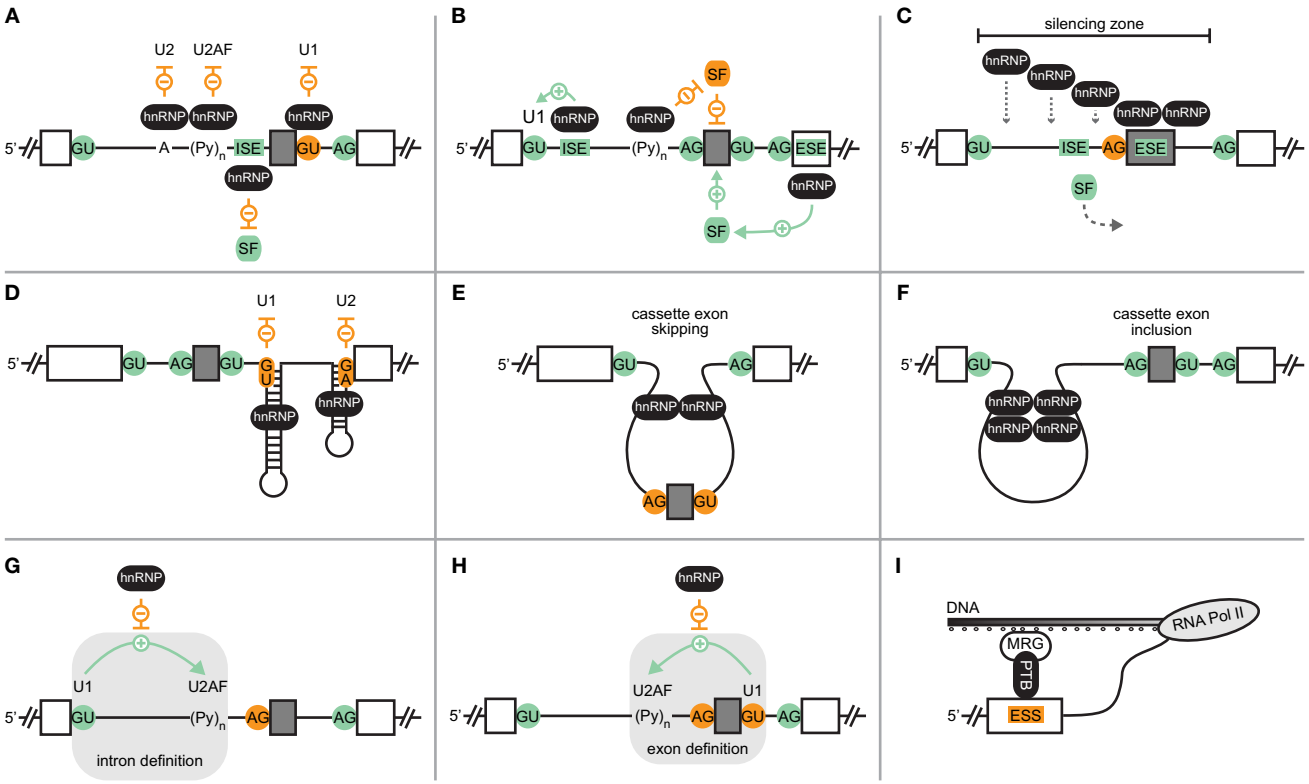

FIGURE 1 | Mechanisms of hnRNP-mediated splicing regulation. (A) Competition of hnRNP proteins with components of the spliceosome (U1, U2, U2AF) or a positive splicing factor (SF) results in skipping of a cassette exon (gray box). Exons and introns are depicted as boxes and lines, respectively. Negative interactions and disabled splice sites are shown in orange, positive interactions and activating factors or elements are in green. ISE, intronic splicing enhancer. Symbols and color code apply to all panels. **(B)** Activation of cassette exon inclusion by hnRNP-mediated recruitment of spliceosomal components or positive factors, or by displacement of a negative factor. ESE, exonic splicing enhancer. **(C)** Cooperative binding of hnRNP proteins establishes a zone of silencing, inducing cassette exon skipping. **(D)** Occlusion of splice sites by RNA structures stabilized by hnRNP proteins. **(E)** hnRNP-mediated looping of the cassette exon prevents its inclusion, whereas looping of a region upstream of the cassette exon can bring splice sites together and induce exon inclusion **(F)**. **(G)** Interference of an hnRNP protein with intron definition interactions between U1 and downstream bound U2AF, or inhibition of exon definition **(H)**. **(I)** Histone modification (small circles below DNA)-dependent recruitment of the hnRNP protein PTB via MRG to an exonic splicing silencer (ESS).

of 5′ or 3′ splice sites, branch point and polypyrimidine tract, or binding sites of activating SR proteins. For example, hnRNP L has been shown to suppress the recognition of either 5′ or 3′ splice sites by binding to adjacent, intronic CA-rich motifs (Heiner et al., 2010). In contrast, hnRNP L binding to an intronic CA repeat within human endothelial nitric oxide synthase pre-mRNA stimulates splicing from a nearby 5′ splice site (Hui et al., 2003). This might be explained by an hnRNP protein-mediated recruitment of the U1 small nuclear ribonucleoprotein (snRNP) complex (**Figure 1B**), as shown for hnRNP H-regulated splicing of HIV-1 (Caputi and Zahler, 2002). Alternatively, a positive effect might also be caused by displacement of a splicing repressor, as it has been reported for PTB that can antagonize the splicing suppressing function of SRp30c in the hnRNP A1 pre-mRNA (Paradis et al., 2007). Thus, depending on the binding position, hnRNP proteins can displace or recruit spliceosomal core components and other regulatory factors, thereby altering the splicing outcome.

Often, splicing control by hnRNP proteins not only involves a single binding site within the pre-mRNA, but rather several motifs, for which cooperative binding behavior can occur (**Figure 1C**). For example, upon hnRNP A1 binding to a high-affinity site within HIV-1 pre-mRNA, cooperative binding to additional sites was observed (Okunola and Krainer, 2009). The cooperative spreading of hnRNP A1 preferentially takes place in a 3′- to 5′-direction, resulting in unwinding of RNA structures and removal of bound proteins, thereby establishing a zone of silencing.

MODULATION OF PRE-mRNA STRUCTURES BY hnRNP PROTEINS

Several recent studies argue for a more widespread role of pre-mRNA structures in the regulation of splicing (Warf and Berglund, 2010). Eukaryotic riboswitches represent one example, in which alternate RNA structures play an active role in both signal sensing and splicing regulation via controlling splice site accessibility (Wachter, 2010). In most other reported cases of structure-dependent splicing, however, alternate RNA folds are enforced by the binding of proteins, including members of the group of hnRNP proteins. Looping of RNA regions can lead either to activation or suppression of a certain splicing event. Accordingly, negative control often is mediated by occlusion of *cis*-elements that need to be recognized to enable a certain splicing outcome

(**Figure 1D**). In contrast, RNA structures can also disable binding of splicing suppressors, or expose splicing enhancers, resulting in splicing activation. Finally, formation of RNA folds can alter critical distances, thereby influencing the recognition of splice sites by the spliceosomal machinery (**Figures 1E,F**). Interestingly, RNA looping might be stabilized by a single hnRNP protein containing several RRMs, as suggested for PTB, the RRMs 3 and 4 of which were shown to interact with each other on their dorsal faces, resulting in an antiparallel orientation of bound RNA (Oberstrass et al., 2005; Vitali et al., 2006). Structure-dependent AS control was also proposed for hnRNP C, which can trigger cassette exon skipping or inclusion in a binding position-dependent manner (Konig et al., 2010).

INTERFERENCE OF hnRNPs WITH INTERACTIONS BETWEEN SPLICEOSOMAL COMPONENTS

Besides competition with or recruitment of singular factors, hnRNPs can also interfere with protein interactions that are required for the formation of a functional spliceosomal complex. Different steps in spliceosome assembly can be affected, as nicely illustrated by PTB-regulated AS events. On one hand, PTB can suppress intron definition (**Figure 1G**) by preventing the 5′ splice site-dependent assembly of U2AF into the spliceosomal E complex (Sharma et al., 2005). On the other hand, PTB can also inhibit exon definition (**Figure 1H**), as in the example of Fas exon 6 (Izquierdo et al., 2005). The inclusion of this cassette exon is stimulated by an interaction of U1 snRNP at the 5′ splice site and U2AF at the 3′ splice site, downstream and upstream, respectively. Furthermore, splicing can also be controlled in the phase of transition from exon definition to an intron-defined spliceosome, as was proposed based on proteomic analyses of spliceosomal complexes formed in the presence of PTB (Sharma et al., 2008).

COUPLING OF SPLICING WITH OTHER STEPS IN GENE EXPRESSION

Numerous studies have revealed that splicing can occur co-transcriptionally, and, more recently, examples for functional coupling of splicing and transcription have been described (Oesterreich et al., 2011). Importantly, the rate of transcriptional elongation can influence the availability of competing splice sites and other *cis*-regulatory elements. For example, RNA polymerase II pausing can delay transcription of competing splice sites, thereby triggering inclusion of a cassette exon with weak splice sites. Several RNA binding proteins, including PTB, were found to be associated with the transcriptional complex, with striking differences in their distribution along different regions of the genes (Swinburne et al., 2006). Furthermore, a correlation between the association of PTB and RNA polymerase II was revealed, and it was hypothesized that transcriptional complexes containing certain RNA binding proteins might display altered elongation rates, having important implications for AS decisions (Swinburne et al., 2006). Similarly, hnRNP proteins might also interfere with other steps in mRNA processing, such as 3′ end processing, and thereby regulate AS.

Furthermore, a recent study also described a direct link between epigenetic modifications and PTB-dependent splicing regulation in human (Luco et al., 2010). Luco et al. (2010) found a correlation between certain histone modifications and the AS outcome for various genes, which was attributed to the histone mark-specific recruitment of PTB via the chromatin-binding protein MRG15 that serves as adaptor (**Figure 1I**). Based on these findings, it is tempting to speculate that further links between epigenetic modifications and AS might exist.

SPLICING REGULATORY FUNCTIONS OF POLYPYRIMIDINE TRACT-BINDING PROTEINS

Polypyrimidine tract-binding proteins represent one of the best studied families of hnRNPs that fulfill diverse functions in mRNA metabolism (Spellman and Smith, 2006; Sawicka et al., 2008), including regulation of pre-mRNA splicing and polyadenylation, translation of viral RNAs from internal ribosomal entry sites (IRES), and mRNA transport. Numerous reports have highlighted the critical role of PTBs in controlling AS decisions, thereby providing important insights into both mechanistic aspects and functional implications of regulated AS events.

In line with the findings for other splicing regulatory factors, the mode of action of PTB is highly dependent on the binding position within the pre-mRNA. Best studied is splicing inhibition by PTB, resulting in skipping of cassette exons (Spellman and Smith, 2006). For some other AS events, however, a stimulatory effect of PTB on exon inclusion was revealed, as for example in the case of the pre-mRNA of calcitonin and calcitonin gene-related peptide (Lou et al., 1999). To address the binding position-dependent splicing action of PTB, Xue et al. (2009) applied cross-linking immunoprecipitation coupled with high-throughput sequencing (Clip-seq) to identify PTB binding sites within the transcriptome. Based on their findings, it was suggested that PTB binding close to alternative sites generally induces exon skipping, whereas binding close to the constitutive sites typically is linked to inclusion of an alternative exon. This model is in line with the classical view of PTB as a general splicing repressor, where weakening of constitutive splice sites allows inclusion of a cassette exon. However, a different model of position-dependent AS regulation by PTB was put forward by Llorian et al. (2010), who employed high-resolution splice-sensitive microarrays to study AS upon PTB knockdown. A majority of the discovered PTB-dependent AS events were PTB-repressed cassette exons, which typically had PTB binding sites upstream of or within the respective exon. In contrast, PTB-stimulated exons possessed polypyrimidine motifs downstream of the cassette exon, indicating a direct activation of exon inclusion by PTB rather than acting by weakening of neighboring constitutive sites. The discrepancies between these two studies might be explained, at least partially, by the different methods that were applied, as well as by the distinct sets of analyzed AS events. Accordingly, analysis of the Clip-seq data from the study by Xue et al. (2009) with respect to the PTB regulation targets identified by Llorian et al. (2010) further supported the model suggested in the latter work. Moreover, previous studies had highlighted that PTB can regulate splicing via different mechanisms, and, similarly, also the position-dependent splicing outcome might vary between AS events.

Most of the work on the mechanistic aspects of splicing regulation by PTB has focused on the repression of exon inclusion (Spellman and Smith, 2006), which still appears to be the dominant type of PTB-controlled AS. Several of the aforementioned mechanisms of AS control have also been proposed to underlie PTB-mediated splicing control, the studies of which have made major

contributions to our current understanding of regulated splicing. Again, it should be highlighted that these models are not mutually exclusive, but rather might be implemented in the context of different AS events. PTB displays a high-affinity for pyrimidine-rich sequences, and, therefore, can compete with U2AF65 for binding to the polypyrimidine tract (Singh et al., 1995; Sauliere et al., 2006), which is typically present in the region between the intronic branch point and the 3′ splice site. However, given that most pre-mRNAs with PTB-regulated exons contain several PTB motifs, many of which are not located within the polypyrimidine tract (Wagner and Garcia-Blanco, 2001; Amir-Ahmady et al., 2005), additional mechanisms of PTB-mediated AS must exist. Proposed models include propagation of PTB protein binding between high-affinity sites on the pre-mRNA, and RNA looping, both of which can establish a zone of silencing, where *cis*-regulatory elements are masked, leading to skipping of the respective exon (Wagner and Garcia-Blanco, 2001; Spellman and Smith, 2006). Due to its RRM domain organization (Oberstrass et al., 2005; Vitali et al., 2006), PTB might cause RNA looping by binding to two distinct sites within the same pre-mRNA molecule. However, artificial tethering of PTB domains to a pre-mRNA via the phage MS2 RNA-protein interaction system preserved PTB-mediated splicing control, indicating that, at least in some instances, PTB action can be uncoupled from the mode of RNA binding (Robinson and Smith, 2006). Besides direct occlusion of *cis*-elements, PTB was demonstrated to disturb the interaction of protein factors at different steps of spliceosome assembly, thereby suppressing splicing (see above). While several models exist that describe splicing inhibition by PTB, only few studies have addressed the mode of action of this protein as a positive splicing regulator, as in the case of the alternative exon 7B of the hnRNP A1 pre-mRNA (Paradis et al., 2007). Here, the stimulatory effect was found to originate from PTB counteracting the splicing inhibitory activity of the SRp30c protein. Mechanistic studies of further PTB-stimulated exons might reveal if this anti-repressor activity of PTB is more widespread in AS control.

Polypyrimidine tract-binding protein contributes to the regulation of numerous AS events in mammals, as revealed by studies applying splice-sensitive microarrays to compare transcriptome-wide splicing patterns between control and PTB knockdown samples (Boutz et al., 2007; Xing et al., 2008; Llorian et al., 2010). Upon down-regulation of PTB, Llorian et al. (2010), for instance, identified 196 repressed and 67 activated exons, a major fraction of which showed neuronal and striated muscle specificity. Further important insights into biological functions of PTB-controlled AS programs in mammals were gained by analysis of tissue-specific expression of PTB and its neuronal homolog nPTB. In neuronal cells, PTB is down-regulated by a tissue-specific microRNA (Makeyev et al., 2007), leading to a switch from PTB to nPTB, coinciding with an altered splicing program in developing neurons (Boutz et al., 2007; Makeyev et al., 2007). Direct comparison of RNA binding affinities and *in vitro* splicing regulation of PTB and nPTB (Markovtsov et al., 2000), as well as knockdown of individual or in parallel both PTB isoforms followed by transcriptome-wide splicing studies revealed both specific and redundant functions of those proteins (Boutz et al., 2007; Spellman et al., 2007). Interestingly, PTB and nPTB levels are subject to feedback control via auto-regulation, whereas PTB also negatively regulates nPTB expression (Wollerton et al., 2004; Boutz et al., 2007; Makeyev et al., 2007; Spellman et al., 2007). These auto-and cross-regulatory circuits involve formation of splicing variants that are targeted by nonsense-mediated decay (NMD). In addition, evidence for splicing-independent repression of PTB protein generation has been provided, however, the underlying mechanism remained ill-defined. Moreover, cross-regulation of mammalian PTBs is not limited to PTB and nPTB, but also includes the less-studied, hematopoietic cell-specific PTB homolog ROD1, the splicing of which is altered upon simultaneous knockdown of PTB and nPTB (Spellman et al., 2007).

By far most of the work on PTBs was performed in mammalian systems, however, homologs of those proteins are also present and have been studied in other eukaryotic clades. For example, in *Drosophila*, the PTB gene was shown to encode a germline-specific mRNA isoform that is required for male fertility by contributing to spermatid individualization (Robida et al., 2010). Recently, PTBs have also been described in plants (Ham et al., 2009; Wang and Okamoto, 2009) and, in the case of three PTB homologs from *Arabidopsis*, evidence for their splicing regulatory functions has been provided (Stauffer et al., 2010). Ham et al. (2009) identified a pumpkin PTB as a constituent of a phloem-mobile ribonucleoprotein complex, the assembly of which depends on PTB phosphorylation (Li et al., 2011). A study of the two closely related *Arabidopsis* PTB homologs, AtPTB1 and AtPTB2, suggested a role of those proteins in pollen germination, as mutants displayed diminished germination efficiency (Wang and Okamoto, 2009). However, the molecular basis of this phenotype and the mode of action of AtPTB1 and 2 remained unsolved.

The work by Stauffer et al. (2010) delivered the first insights into the splicing regulatory potential of all three *Arabidopsis* PTB homologs, the expression of which is controlled by auto- and cross-regulation, akin to the mammalian PTBs. Analysis of the splicing patterns for all three genes revealed the existence of two major splicing products, of which one encodes the full-length protein, whereas the other contains a premature termination codon (PTC) and is targeted by NMD (**Figure 2**). Splicing to the NMD target transcript can be triggered in an auto-regulatory feedback loop, where an elevated level of one PTB alters splicing of its corresponding pre-mRNA. Additionally, cross-regulation between the two close homologs AtPTB1 and 2, but not the distantly related AtPTB3, was observed. These findings revealed not only the splicing control functions of AtPTBs, but also that regulation of PTBs via auto- and cross-regulatory feedback loops is present in both mammals and plants. Future work will reveal if plant PTBs, as their mammalian counterparts, also contribute to complex splicing programs, and, if so, what the biological implications of those AS events are. AtPTB1 and 2 are close homologs, with more than 80% of their amino acid residues conserved, and, in both cases, retention of a cassette exon with a PTC results in formation of the NMD-targeted splicing variant (Stauffer et al., 2010). In contrast, AtPTB3 is more distantly related, displaying a comparable degree of protein homology of ∼50% to the other two *Arabidopsis* and the human PTB homologs. Furthermore, as for human PTB, feedback regulation of AtPTB3 gives rise to skipping of a cassette exon, resulting in a downstream PTC and NMD targeting. Thus, AS of plant PTB pre-mRNAs can serve as a model to study both

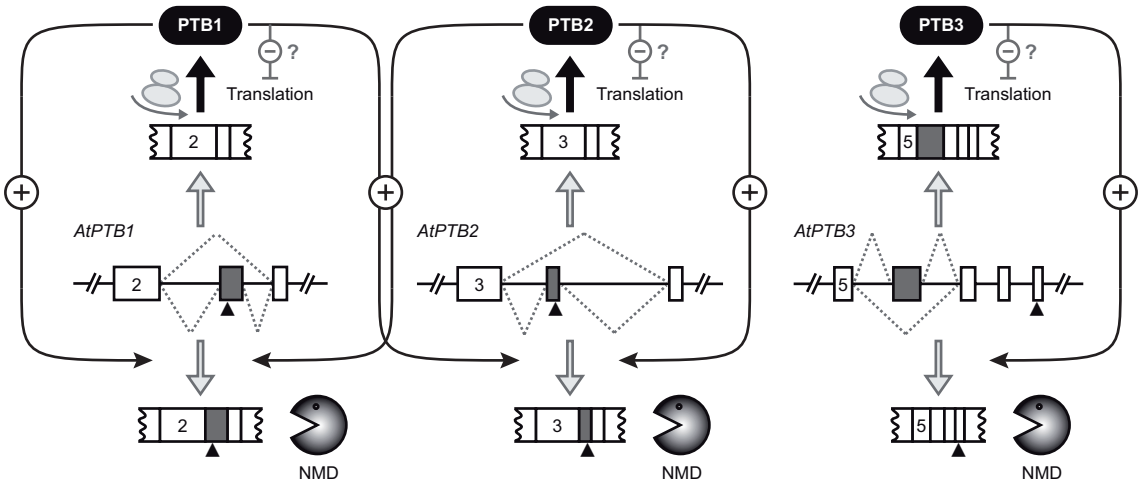

FIGURE 2 | Auto- and cross-regulation of the *Arabidopsis PTB* homologs. Exon-intron organizations of the *Arabidopsis genes AtPTB1*, *AtPTB2*, and *AtPTB3* are shown for the regions giving rise to alternative splicing (dotted lines). Exons and introns are depicted as boxes and lines, respectively. Gray boxes refer to cassette exons, which are either skipped or included. Numbers of first displayed exons are given for all three genes. Black triangles indicate premature termination codons, rendering the respective splicing variants degradation via nonsense-mediated decay (NMD, bottom). The splicing variants shown on top of each gene model are translated into PTB proteins, which can interfere with translation of *PTB* mRNAs (for simplicity, only the effect on their own mRNAs is shown). PTB proteins also trigger splicing of their own pre-mRNAs to the NMD target variants (auto-regulation). Additionally, cross-regulation between *PTB1* and *PTB2* occurs. The model is based on the work from Stauffer et al. (2010).

positive and negative effects of PTBs on exon inclusion. Interestingly, *in vivo* splicing reporter assays indicated that AtPTB2- and AtPTB3-mediated exon inclusion and skipping, respectively, can be counteracted by elevated levels of U2AF65 (Stauffer et al., 2010). These findings are in line with competition between PTBs und U2AF65 in splice site choice, but further work is needed to elucidate the molecular basis of those findings.

Auto- and cross-regulation of *Arabidopsis* PTB homologs does not only affect AS, but also acts downstream of it. Co-expression of PTBs with reporter constructs containing the 5′ region of mature PTB mRNAs resulted in reduced accumulation of the translation products, whereas processing and levels of the corresponding transcripts appeared unchanged (Stauffer et al., 2010). Interestingly, *Arabidopsis* PTBs fused to fluorescent proteins were found to localize to the nucleus, cytosol, and processing bodies (P-bodies). Thus, as an intriguing possibility, PTBs might interfere with expression of their own and also other mRNAs by retracting them from the polysomal pool, followed by storage in P-bodies. Splicing-independent functions of PTBs have also been described in other species, including repression of translation in *Drosophila* (Besse et al., 2009) and human (Boutz et al., 2007), as well as the widespread positive role in translation from IRES (Auweter and Allain, 2008; Sawicka et al., 2008). Further work will be required to understand those diverse regulatory functions of PTBs in more detail.

SPLICING REGULATION AND FURTHER FUNCTIONS OF OTHER PLANT hnRNP PROTEINS

Besides PTB homologs, several other types of hnRNP proteins from different plant species have been investigated. These studies provided important insights into the mode of action and biological functions of this versatile group of proteins, and, given that many other hnRNP homologs from plants still are at best partially characterized, an even wider scope of hnRNP-dependent functions in plants can be anticipated. As reported for their animal counterparts, hnRNP proteins from plants can regulate splicing, but also affect other nucleic acid metabolic processes, a brief summary of which will be provided in the following sections.

REGULATION OF ALTERNATIVE SPLICING BY AtGRP7 AND AtGRP8

AtGRP7 and AtGRP8 are glycine-rich RNA binding proteins (GRPs) from *Arabidopsis* that regulate AS of their corresponding pre-mRNAs in auto- and cross-regulatory circuits (Staiger et al., 2003; Schoning et al., 2008) in a similar manner as described in the previous section for PTBs. Constitutive overexpression of AtGRP7 promotes usage of a cryptic splice site within an intron of the pre-mRNA derived from the endogenous *AtGRP7* locus, giving rise to a splicing variant with a decreased half-life (Staiger et al., 2003). The authors further demonstrated that AtGRP7 overexpression affects the AS of the *AtGRP8* pre-mRNA, which encodes an RNA binding protein closely related to AtGRP7 and exhibits a cryptic splice site conserved with that of *AtGRP7*. Similarly to the *AtGRP7* regulatory circuit, AtGRP8 can regulate splicing of its own and the *AtGRP7* pre-mRNA by triggering the use of an alternative splice site (Schoning et al., 2008). Both AS variants derived from *AtGRP7* and *AtGRP8* harbor PTCs, targeting them for rapid degradation via NMD, as confirmed by their accumulation in NMD-impaired mutants and upon cycloheximide treatment (Staiger et al., 2003; Schoning et al., 2007). By promoting unproductive splicing coupled to a decay of transcripts via the NMD pathway, the interlocked regulatory circuits might be a means to integrate diverse stimuli, thereby fine tuning the expression of their components, and even to influence common downstream targets. Moreover, Schoning et al. (2007) could substantiate that the binding of AtGRP7 to its

target transcripts is necessary for the described negative regulation. By introducing a single arginine to glutamine point mutation into the AtGRP7 RRM, the *in vitro* RNA binding affinity of the protein was reduced, disrupting the AtGRP7-mediated auto-regulation as well as the regulation of downstream targets *in vivo* (Schoning et al., 2007).

AtGRP7 has been demonstrated to be regulated by the circadian clock and contributes to the control of flowering time. Both, an *atgrp7* T-DNA insertion mutant, and independent RNA interference lines with reduced levels of AtGRP7 and the closely related AtGRP8, showed a late flowering phenotype, whereas *AtGRP7* overexpressing plants were reported to flower early (Streitner et al., 2008). Changes in flowering time were found to be mediated by altered transcript levels of the key regulator FLOWERING LOCUS C (FLC), displaying elevated, and diminished *FLC* transcript levels, respectively, in AtGRP7 loss-of-function and overexpression mutants (Streitner et al., 2008). Interestingly, disturbing AtGRP7 levels did not interfere with the photoperiodic response and the effects on flowering time were overridden by vernalization, both of which are features of the autonomous pathway.

To gain further insights into the functions of AtGRP7, global transcript profiles of an overexpression mutant in comparison to wild type plants were analyzed (Streitner et al., 2010). Thereby, around 300 transcripts showing altered levels upon *AtGRP7* overexpression were found, of which one third is under control of the circadian clock. Additionally, transcripts responsive to abiotic and biotic stimuli were identified as putative targets of AtGRP7, as well as components involved in ribosome function and RNA metabolism, consistent with its role in post-transcriptional regulation. These findings suggest a complex set of AtGRP7 regulation targets, the number of which might further increase, if splicing-sensitive transcriptome studies would be applied. An involvement of AtGRP7 in diverse biological processes is also supported by the altered performance of misexpression lines under biotic and abiotic stress conditions. AtGRP7 plays a role in plant immunity, as knockout of this hnRNP resulted in increased susceptibility to the pathogen *Pseudomonas syringae* (Fu et al., 2007). Fu et al. (2007) found that suppression of plant immunity by *P. syringae* involves a mono-ADP-ribosyltransferase as an effector protein, which modifies RNA binding proteins including AtGRP7. Recently, Jeong et al. (2011) identified a conserved arginine residue within the RRM of AtGRP7 as the site of ADP-ribosylation, resulting in diminished RNA binding affinity *in vitro*. Interestingly, earlier studies had revealed that mutation of this residue also disrupts the activities of AtGRP7 *in vivo* (Schoning et al., 2007), highlighting its critical role in protein functioning. In addition to its role under biotic stress, AtGRP7 also has been reported to play a role in seed germination, seedling growth, and under various abiotic stress conditions, including high salt and freezing (Kim et al., 2008b).

ARGININE METHYLATION AFFECTS AS IN PLANTS

Arginine methylation occurs not only in histones, but also has been described for many other protein classes, including transcription factors and certain types of hnRNP proteins (Lee and Stallcup, 2009). In *Arabidopsis*, mutation of the *arginine methyltransferase 5* (*AtPRMT5*) was found to cause pleiotropic phenotypes, including late flowering, growth retardation, and a reduced vernalization sensitivity (Pei et al., 2007; Wang et al., 2007; Schmitz et al., 2008). Deng et al. (2010) demonstrated that AtPRMT5 not only methylates histones but also has a variety of non-histone substrates, including RNA binding or processing factors, like the hnRNPs AtGRP7 and AtGRP8, as well as several U snRNP core proteins. Using a high-throughput sequencing approach, hundreds of genes involved in multiple biological processes, displaying splicing changes upon mutation of *AtPRMT5* were identified (Deng et al., 2010). Based on these results, the authors could attribute the late flowering phenotype of *atprmt5* mutants to AS of transcripts encoding for RNA processing factors involved in flowering time regulation (**Figure 3**). In particular, the described AS event resulted in decreased levels of the transcript variant encoding the functional form of the hnRNP E homolog FLK, an autonomous pathway protein known to promote flowering by repressing the expression of *FLC* (Lim et al., 2004; Mockler et al., 2004). Therefore, in addition to regulating transcription through histone modifications, AtPRMT5 may contribute directly or indirectly to the regulation of pre-mRNA splicing through modifications of non-histone proteins.

In another study of the same arginine methyltransferase, Sanchez et al. (2010) showed that AtPRMT5 also provides a link between AS and the circadian clock (**Figure 3**). In line with the results obtained by Deng et al. (2010), Sanchez et al. (2010)

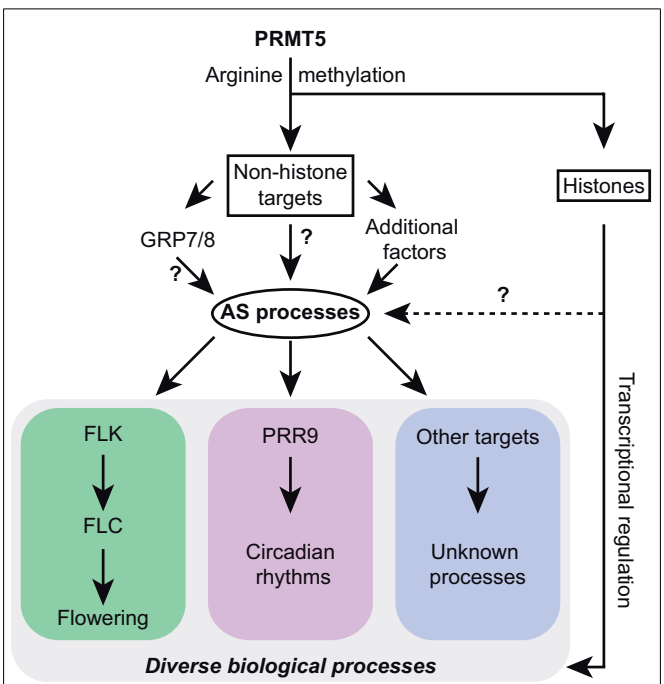

FIGURE 3 | Arginine methylation and alternative splicing are linked in *Arabidopsis*. The arginine methyltransferase PRMT5 modifies both histone and non-histone targets, including the splicing factors GRP7 and GRP8 (Deng et al., 2010). Changes in alternative splicing (AS) affect diverse biological processes, such as FLK/FLC-mediated flowering control (Deng et al., 2010) and regulation of circadian rhythms via PRR9 (Sanchez et al., 2010). A connection between histone modifications and AS has been demonstrated in mammals (Luco et al., 2010), but so far not in plants.

uncovered numerous splicing changes in the *atprmt5* mutant, including AS of the core-clock gene *PSEUDO RESPONSE REGULATOR 9* (*PRR9*). The altered splicing of *PRR9* might at least partially explain the impairment of several circadian rhythms in the *atprmt5* mutant. Interestingly, the expression of *AtPRMT5* underlies circadian oscillations, which are reflected in expression and splicing patterns of a subset of genes. The authors further found an enrichment of alternative 5′ splice sites among the splicing events altered in the *atprmt5* mutant, indicating a role of this factor in 5′ splice site recognition. Thus, analyses of splicing patterns in *atprmt5* mutants provided an intriguing link between this protein and AS control in plants. However, the molecular mechanisms of this post-transcriptional regulation as well as further physiological processes that AtPRMT5 might be involved in, remain to be uncovered.

UBP1 AND UBP1-ASSOCIATED PROTEINS

The oligouridylatebinding protein 1 (UBP1) from *Nicotiana plumbaginifolia* has been characterized as a nuclear RNA binding protein that associates with polyA-RNA *in vivo* and can be cross-linked to U-rich intron and UTR sequences *in vitro* (Lambermon et al., 2000). UBP1 was found to enhance the splicing efficiency of otherwise inefficiently processed introns, as well as to increase the accumulation of reporter mRNAs that are intronless or bear suboptimal introns. Only the UBP1-mediated accumulation of reporter mRNAs was shown to be promoter dependent, suggesting independent activities of UBP1 at more than one level of plant nuclear pre-mRNA maturation. UBP1 homologs are also present in *Arabidopsis*, however, so far no splicing functions of these proteins have been reported. In a follow-up study by Lambermon et al. (2002), two UBP1-associated proteins, UBA1a and UBA2a, were identified that also can stabilize mRNAs in the nucleus. However, in contrast to UBP1, these hnRNP proteins show no effect on pre-mRNA splicing. Expression studies of three *UBA2* genes from *Arabidopsis* revealed AS of their corresponding pre-mRNAs, which is regulated in response to wounding and alters the 3′ UTRs of the respective transcripts (Bove et al., 2008).

Further investigations of hnRNP proteins from *N. plumbaginifolia* resulted in the identification of the two structurally related proteins RBP45 and RBP47, which display, similar to UBP1, specificity for oligouridylates (Lorkovic et al., 2000). RBP45 and RBP47 are also localized in the nucleus and associate with polyA-RNA. However, no stimulation of splicing or accumulation of mRNAs could be observed upon overexpression of *RBP45* and *RBP47* in a protoplast system (Lorkovic et al., 2000), suggesting a participation of these proteins in a different step of pre-mRNA maturation. Alternatively, the absence of splicing effects might also be explained by saturating levels of the respective factors already without their overexpression in this particular experimental system, and, thus, does not generally exclude a role of those proteins in splicing. In line with this hypothesis, both UBP1 and RBP45 were able to enhance intron recognition upon overexpression in an artificial mini-exon system (Simpson et al., 2004). Furthermore, in an approach to determine proteins affecting plant U12-dependent intron splicing, no effect of RBP45 or UBP1 on splicing efficiency for this rare intron type was observed (Lewandowska et al., 2004).

PLANT hnRNP PROTEINS WITH UNKNOWN OR SPLICING-UNRELATED MODES OF FUNCTION

Biological roles have been ascribed to several other plant hnRNP proteins, e.g., by analyzing biological consequences upon altering their expression. The mode of action for many of these factors, however, remains largely unknown. For example, the AAPK-interacting protein 1 (AKIP1) of *Vicia faba*, a protein with sequence homology to hnRNP A/B, was revealed to be a substrate of the abscisic acid-activated protein kinase (AAPK; Li et al., 2002). This kinase is localized in guard cells, where it regulates plasma membrane ion channels in response to the stress hormone abscisic acid (ABA). Both AKIP1 and AAPK were shown to be nuclear localized, but upon *in vivo* treatment with ABA, AKIP1 increasingly localized to speckle-like structures within the nucleus. Additionally, upon ABA-mediated phosphorylation of AKIP1 by AAPK, AKIP1 displayed an increased affinity for its interaction target, the mRNA of dehydrin, a protein reported to be involved in cell protection under stress conditions. As mentioned before, post-translational modifications have been described for several other hnRNP proteins and, thus, might play a more widespread role in defining their RNA target specificity.

The closest homolog of *V. faba* AKIP1 is the previously described protein UBA2a from *Arabidopsis*, showing a similar nuclear reorganization to speckles in response to ABA (Riera et al., 2006). However, in contrast to AKIP1, UBA2a is, if at all, only weakly phosphorylated by OPEN STOMATA 1 (OST1), the *Arabidopsis* ortholog of AAPK. Overexpression of the three *Arabidopsis UBA2a* genes was demonstrated to be lethal, while expression under control of an inducible system triggered leaf senescence and hypersensitive-like cell death (Kim et al., 2008a). Further work will be required to reveal if these phenotypes are linked to altered metabolism of distinct target transcripts.

A possible link between polycomb regulation, which is based on polycomb complex-mediated control of gene expression via modulation of epigenetic patterns (Schwartz and Pirrotta, 2007), and RNA processing was indicated by investigation of LIF2, a putative RNA binding protein of the hnRNP family. LIF2 interacts with the chromo domain protein Like Heterochromatin Protein 1 (LHP1; Latrasse et al., 2011), which is a subunit of a polycomb repressive complex (PRC) in *Arabidopsis*. LHP1 recognizes histone H3 lysine 27 trimethylation, an epigenetic silencing signal, deposited by the PRC2 complex. Its interaction partner LIF2 was described to be able to either antagonize or act with LHP1, suggesting that it may modulate LHP1-activity at specific loci or in response to environmental changes, in order to control cell fate determination.

Besides their function in RNA metabolism, hnRNP proteins can also bind to DNA and affect diverse processes, such as replication, DNA repair, and transcription. One example from plants is the G-strand specific single-stranded telomere binding protein GTBP1 from *Nicotiana tabacum*, which negatively regulates telomere length (Lee and Kim, 2010). Using an RNA interference approach to downregulate expression of *GTBP1*, Lee and Kim (2010) found severe developmental abnormalities in the mutant plants, as well as signs of genome instability, including longer telomeres, formation of extrachromosomal telomeric circles and abnormal anaphase bridges. Subsequently, they could reveal a function of GTBP1 in inhibiting telomeric strand invasion,

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