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Positioning of pyrimidine motifs around cassette exons defines their PTB-dependent splicing in Arabidopsis

Rica Burgardt[1], Dorothee Lambert[2], Christina Heuwieser[2], Maximilian Sack[3], Gabriele Wagner[2], Zasha Weinberg[3] and Andreas Wachter[1,2,*]

[1]*Institute for Molecular Physiology (imP), University of Mainz, Hanns-Dieter-Hüsch-Weg 17, 55128 Mainz, Germany,*
[2]*Center for Plant Molecular Biology (ZMBP), University of Tübingen, Auf der Morgenstelle 32, 72076 Tübingen, Germany, and*
[3]*Bioinformatics Group, Department of Computer Science and Interdisciplinary Centre for Bioinformatics, Leipzig University, Härtelstraße 16-18, 04107 Leipzig, Germany*

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*For correspondence (e-mail wachter@uni-mainz.de).

SUMMARY

Alternative splicing (AS) is a complex process that generates transcript variants from a single pre-mRNA and is involved in numerous biological functions. Many RNA-binding proteins are known to regulate AS; however, little is known about the underlying mechanisms, especially outside the mammalian clade. Here, we show that polypyrimidine tract binding proteins (PTBs) from *Arabidopsis thaliana* regulate AS of cassette exons via pyrimidine (Py)-rich motifs close to the alternative splice sites. Mutational studies on three PTB-dependent cassette exon events revealed that only some of the Py motifs in this region are critical for AS. Moreover, *in vitro* binding of PTBs did not reflect a motif's impact on AS *in vivo*. Our mutational studies and bioinformatic investigation of all known PTB-regulated cassette exons from *A. thaliana* and human suggested that the binding position of PTBs relative to a cassette exon defines whether its inclusion or skipping is induced. Accordingly, exon skipping is associated with a higher frequency of Py stretches within the cassette exon, and in human also upstream of it, whereas exon inclusion is characterized by increased Py motif occurrence downstream of said exon. Enrichment of Py motifs downstream of PTB-activated 5′ splice sites is also seen for PTB-dependent intron removal and alternative 5′ splice site events from *A. thaliana*, suggesting this is a common step of exon definition. In conclusion, the position-dependent AS regulatory mechanism by PTB homologs has been conserved during the separate evolution of plants and mammals, while other critical features, in particular intron length, have considerably changed.

Keywords: alternative splicing, RNA-binding protein, RBP, Polypyrimidine tract binding protein, PTB, *Arabidopsis thaliana*.

INTRODUCTION

Precursor mRNA (pre-mRNA) molecules undergo extensive co- and post-transcriptional processing in the nucleus as a prerequisite for the generation of mature and export-competent mRNAs. RNA processing also plays a pivotal role in augmenting transcriptome complexity by creating transcript variants, for example, via alternative pre-mRNA splicing (AS) based on the various definitions of exonic and intronic regions. Indeed, the majority of intron-containing genes from higher eukaryotes generate AS variants, for example, as demonstrated for *Arabidopsis thaliana* (Marquez et al., 2012; Zhang et al., 2022), involving complex regulatory mechanisms and contributing to diverse biological functions (Reddy et al., 2013; Staiger & Brown, 2013). Deciphering the splicing code underlying this specific AS regulation remains a major challenge, in particular in plants, despite substantial progress made in the past two decades with regard to the characterization of individual AS regulatory components and mechanisms. New techniques such as iCLIP (individual nucleotide UV cross-linking immunoprecipitation; Burjoski & Reddy, 2021; Hafner et al., 2021) and TRIBE (targets of RBPs identified by editing; McMahon et al., 2016) provided transcriptome-wide information about the targets and binding motifs of individual RNA-binding proteins (RBPs). However, these analyses need to be complemented by functional studies, also to be able to evaluate the consequences of the corresponding interactions. Accordingly,

transcriptome-wide mapping of the RNA binding sites for two archetypical splicing regulators from mouse was found to be insufficient to explain their impact on AS (Pandit et al., 2013).

In plants, comparatively little is known about the splicing regulatory code and only a few studies linked individual *cis*-elements to a specific AS outcome. For example, Yoshimura et al. (2002) identified a conserved intronic motif that is responsible for tissue-specific AS of the pre-mRNA from the chloroplast ascorbate peroxidase. Using a splicing reporter approach in *A. thaliana*, Thomas et al. (2012) demonstrated that an intronic element with several GAAG repeats is required for proper AS of the *SCL33* pre-mRNA. In case of AtGRP7 and its homolog AtGRP8, auto- and cross-regulation of their expression via AS coupled to nonsense-mediated decay (NMD) was shown (Schöning et al., 2007, 2008) and nucleotides within the *GRP7* intron sequence that are critical for the *in vitro* binding of the two proteins were identified (Leder et al., 2014). The use of iCLIP resulted in the identification of 2705 transcripts with AtGRP7 binding sites (Lewinski et al., 2024; Meyer et al., 2017), and a conserved U-rich motif was shown to be bound by the protein *in vitro*.

Among the best-studied splicing regulators are the POLYPYRIMIDINE TRACT BINDING PROTEINS (PTBs) that belong to the family of heterogenous nuclear ribonucleoprotein proteins (hnRNPs) and are present in animals and plants (Sawicka et al., 2008; Wachter et al., 2012). In vertebrates, several PTB homologs exist and activation of a neural PTB provokes a specific AS program contributing to neuronal differentiation (Boutz et al., 2007; Gueroussov et al., 2015; Li et al., 2014). PTB has been demonstrated or proposed to alter alternative splice site choice by various means, such as competing with the binding of U2AF65, interfering with the assembly of spliceosomal complexes, and forming RNA loops (Sawicka et al., 2008; Spellman & Smith, 2006; Wachter et al., 2012). *In vitro* studies, genetics, and CLIP-seq identified pyrimidine (Py)-rich stretches with alternating C and U nucleotides as preferred binding sites of human PTB (Oberstrass et al., 2005; Pérez et al., 1997; Reid et al., 2009; Xue et al., 2009). Moreover, the splicing activity of PTB was reported to depend on the binding position (Llorian et al., 2010; Xue et al., 2009). Interestingly, these two studies came to different conclusions. While Xue et al. (2009) proposed that PTB binding in vicinity of facultative cassette exons triggers their skipping, the opposite effect, namely increased inclusion, was linked to PTB binding directly downstream of the cassette exon in the report by Llorian et al. (2010).

PTB homologs are present throughout the plant kingdom, with three *PTB* genes in *A. thaliana* (Stauffer et al., 2010). AtPTB1 and AtPTB2 are closely related and auto- and cross-regulate their expression via triggering the inclusion of cassette exons that introduce premature termination codons (PTCs) and thereby cause NMD targeting. AtPTB3 is more distantly related to the other two proteins and negatively regulates only its own pre-mRNA via AS; in this case by skipping of a coding exon, which results in a frameshift and a downstream PTC. Negative auto- and cross-regulation via skipping of a coding exon and subsequent NMD targeting were also demonstrated for mammalian *PTB* genes (Spellman et al., 2007; Wollerton et al., 2004). Transcriptome-wide AS studies identified ~450 AS events being responsive to the levels of PTB1 and PTB2 in *A. thaliana*, whereas in case of PTB3, no additional AS targets were found (Rühl et al., 2012). Cassette exons were overrepresented among the AtPTB1/2-regulated events, with most of them being included in a PTB-dependent manner. Simpson et al. (2014) confirmed the role of AtPTB1/2 in AS regulation and demonstrated their ability to repress a mini exon in an artificial splicing reporter. *In vitro* binding of recombinant AtPTB2 to an RNA fragment corresponding to the PTB-regulated cassette exon of *PIF6* with flanking intronic sequences including several Py-rich stretches was reported (Rühl et al., 2012). However, the functional relevance of individual Py stretches for PTB-dependent AS control so far has not been tested for any natural regulation target in *A. thaliana*.

The characterization of misexpression lines provided evidence for the role of PTB1 and PTB2 in regulating seed germination and the expression of flowering regulators in *A. thaliana* (Rühl et al., 2012). These two PTBs were also reported to function in pollen germination (Wang & Okamoto, 2009). Given the large number of AS targets, an even wider role of AtPTB1/2 in regulating physiological processes can be anticipated. PTB3 homologs from pumpkin (Ham et al., 2009) and potato (Cho et al., 2015) were identified as constituents of phloem-mobile RNPs, suggesting functional specialization among PTB homologs in plants. Understanding the molecular basis that defines PTBs' interaction with and regulation of target RNAs will be important to decipher their functional role in the splicing code and likely beyond in plants.

In this study, we demonstrate that cassette exon splicing by AtPTB1 and AtPTB2 depends on Py motifs in the vicinity of the alternative splice sites. By combining mutational studies on individual AS events with global bioinformatic analyses, we observe characteristic distribution patterns of Py stretches within cassette exons and their flanking introns that are linked to the PTB-dependent outcome of the corresponding AS event. Such a position-dependent mode of regulation is consistent with reports on other hnRNPs from the animal kingdom and suggests that PTB proteins have retained their regulatory mechanisms over long evolutionary distances.

RESULTS

Exon inclusion by PTB1/2 depends on Py motifs downstream of the cassette exon

Rühl et al. (2012) previously found PTB1 and PTB2 to control at least 452 AS events in more than 300 genes. To determine how this control is conferred on the molecular level, we further characterized several PTB-dependent cassette exon events. As a first model, we selected the *AtPTB2* gene which is negatively auto- and cross-regulated by PTB2 and PTB1 via the inclusion of an alternative exon containing a PTC (Stauffer et al., 2010). Based on the established preference of PTBs for Py motifs, we searched the cassette exon and its flanking introns for Py stretches of at least 5 nt (Figure 1a). To determine which of them are critical for the AS outcome, we used a previously established *PTB2-GFP* splicing reporter (Stauffer et al., 2010; Figure 1b). Candidate binding motifs were mutated individually or two at a time, all Py residues being changed to purines (U to A and C to G), and the splicing response of the resulting reporters in presence or absence of PTB2 was examined in a transient expression system in *Nicotiana benthamiana*. As an output, we used reporter fluorescence: since the SPII splicing variant contains a PTC and is degraded via NMD (Stauffer et al., 2010), fluorescence directly correlates with SPI levels. Of the 13 motifs examined, six resulted in a diminished reporter response to PTB2 presence when mutated (Figure 1c; Figure S1a–c). The most pronounced effect was observed upon mutation of Py3 and Py4, which are located downstream and in vicinity of the cassette exon (Figure 1a). We were able to confirm this result by examining reporter splicing in stable *A. thaliana* lines: mutation of Py2, 3, 4, or 5, all of which are located within ~75 bp downstream of the cassette exon, caused a shift in the splicing variant ratio towards the PTB-independent exon skipping variant (Figure 1d,e). Py1 and 2 additionally seem to have a function in intron definition, as their mutation led to alternative splice site choice in the flanking introns, resulting in CE inclusion variants with partial or complete intron retention (Figure S1d). This might be a consequence of diminished levels of uridines in the introns due to mutation of the corresponding Py motifs to purines, based on previous reports that U-rich sequences are important for intron definition and efficient splicing (Ko et al., 1998; Lou et al., 1993; Simpson et al., 2004). Motif length did not correlate with its impact on AS upon mutation. Also, some of the motifs might act interdependently, as evidenced by a stronger AS shift of the M4M5 double mutation compared to the individual mutants.

The strong autoregulation of *PTB2* pre-mRNA, with less than 10% reporter activity in the presence of PTB2 (Figure S1a; Stauffer et al., 2010), suggested that this AS event is solely or at least mainly regulated by PTB1/2. Therefore, we wanted to investigate binding motifs in other PTB1/2 targets that might be subject to a less stringent control. We first looked at *PIF6*, where PTB1/2 also mediates the inclusion of a cassette exon and which was previously linked to the regulation of seed germination (Figure 2a; Rühl et al., 2012). We constructed a splicing reporter for this gene (Figure 2b) and analyzed how mutation of candidate motifs impacted AS. Since splicing of the *PIF6* reporter was not altered upon PTB2 co-expression in *N. benthamiana* (for further details see Figure 5), all analyses were carried out in stable *A. thaliana* lines. In seedlings, only motif Py2, located 18 nt downstream of the cassette exon, turned out to be critical for exon inclusion (Figure 2a–d). Because previous analyses had shown that AS of *PIF6* differs between tissues (Rühl et al., 2012), we wanted to investigate if the Py motifs might play a role in conferring this specificity. In line with previous results, we obtained a lower amount of SPII relative to SPI in flower buds than in seedlings (Figure 2c,e). As the reporter mimics the splicing behavior of the endogenous gene, any putative regulator of tissue-specific AS must be present upstream or shortly downstream of the AS site to be still included in the reporter (Figure 2b). However, in either tissue, mutation of any Py motif other than Py2 did not result in a substantial change of the splicing ratio, and the effect of M2 is comparable in both cases (Figure 2d,f), indicating that Py2 is the only motif critical for AS in both seedlings and flower buds. Still, Py1, 3, and 4 might have a function in this AS event: The M3M4 double mutation and particularly the simultaneous removal of all four motifs led to the production of additional, larger splice variants due to intron retention (Figure 2c,e), which implies that at least one of them is needed for correct intron definition in this region. This is again consistent with an important role of U-rich sequences in defining introns for their efficient splicing (see previous paragraph).

Given that only some of the candidate Py motifs are critical for AS, we wanted to test if these are also exclusively bound by PTBs. *In vitro* interaction between PTB2 and the *PIF6* RNA was previously demonstrated using electrophoretic mobility shift assay (EMSA; Rühl et al., 2012), and we extended this experiment by analyzing the binding capacity of Py mutants. As expected, PTB2 binds to the WT *PIF6* RNA, indicated by the upshift of the RNA signal in the presence of PTB2 and the strong competition of this shift upon adding an excess of unlabelled WT *PIF6* RNA (Figure 2g,h). Mutated versions of PTB2 and the thioredoxin domain, which is also part of the recombinant PTB2 proteins, did not show RNA binding (Figure S2). Interestingly, *PIF6* RNAs mutated in M1 or M2 are not able to efficiently compete with the WT RNA regarding PTB2 binding (Figure 2g,h). This effect is even more pronounced in the M3 and M4 mutants, as upon addition of the corresponding competitor RNAs less of the upper signal shifts back to

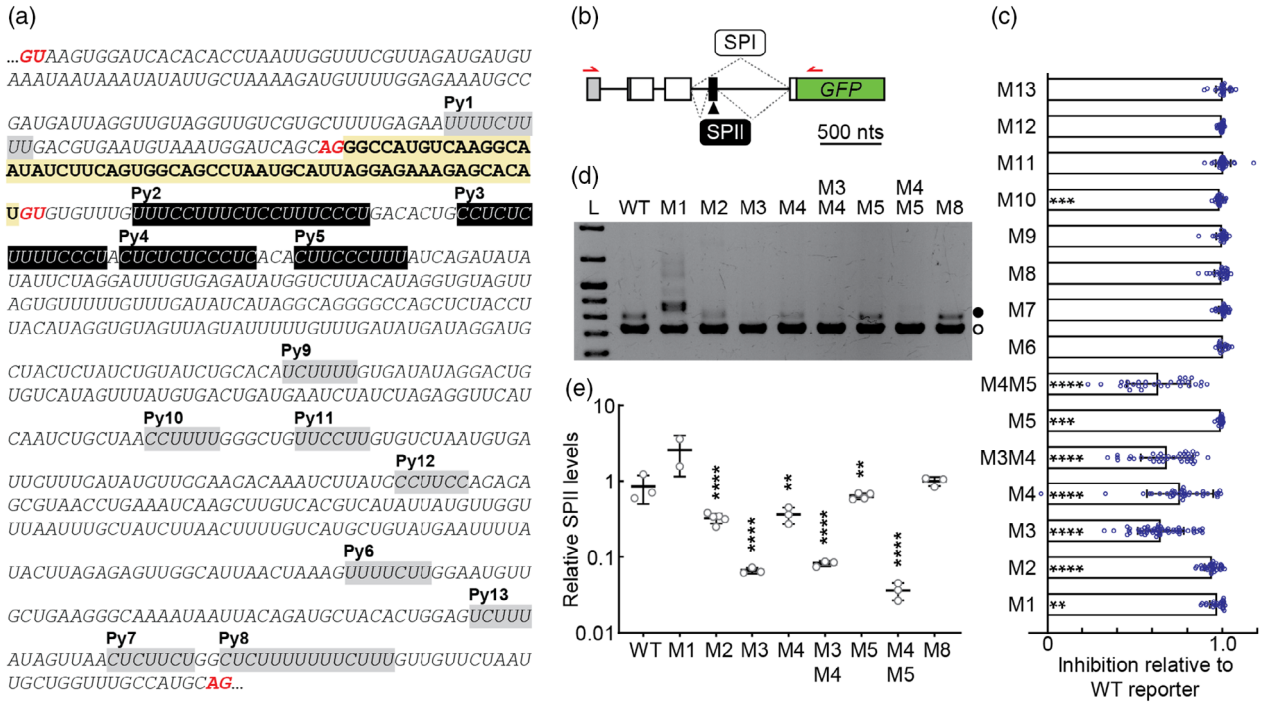

Figure 1. Motifs adjacent to cassette exon control splicing of *AtPTB2* pre-mRNA.
(a) *AtPTB2* sequence including the introns (italics) directly up- and downstream of the cassette exon (bold, highlighted in yellow). Splice sites (red) and pyrimidine-rich stretches (Py, black/gray background) are highlighted.
(b) Splicing reporter covering *AtPTB2* genomic region from 5′ UTR (gray) to partial first exon downstream of cassette exon (black) fused with *GFP* cds. Splicing to SPI and SPII depicted. Exons, introns, premature termination codon, and primer binding sites shown as boxes, lines, triangle, and red arrows, respectively.
(c) AtPTB2-mediated inhibition of mutant reporter constructs [mutant naming corresponds to the numbering of pyrimidine-rich stretches from (a)] relative to a WT sequence version upon transient expression in *N. benthamiana*. Value of 1 indicates the same inhibition as WT reporter. Displayed are mean values, standard deviations, and individual data points; asterisks indicate significant change compared to the WT reporter (one-sample *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).
(d) WT and mutant reporter splicing in stable *A. thaliana* lines. L, size ladder (from bottom: 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, and 1.5 kb). White and black dot indicates the position of SPI and SPII, respectively.
(e) Quantitative analysis of relative SPII levels normalized to one sample from the WT reporter line in stable *A. thaliana* mutants. Displayed are mean value (black line), standard deviation, and individual data points; asterisks indicate significant change compared to the WT reporter (one-sample *t*-test, $^{**}P < 0.01$, $^{****}P < 0.0001$).

the position of the free probe (Figure 2h). The weakest competition is seen with a quadruple mutant construct, in which PTB2 binding is almost completely abolished. Surprisingly, the double mutants M1M2 and M3M4 were less impaired in binding than the corresponding single mutant constructs. This might be caused by the formation of an RNA loop upon base-pairing between the two purine and two Py motifs in the respective double mutant RNAs. As human PTB also binds double-stranded RNA (Spriggs et al., 2005) this putative stem might cause improved binding to the remaining Py motifs. Also interesting is the more pronounced upshift of the RNA signal with increasing concentrations of competitor RNA that is present with the WT sequence or the two single mutants M1 and M2 as competitors, but not for the quadruple mutant (Figure 2g). It might be caused by PTB2 binding to multiple RNA molecules in the presence of an excess of RNA. This would explain why the effect is gone when M1-4 RNA is used as a competitor, as it has probably lost its affinity for PTB2 binding. In conclusion, deletion of any of the four Py motifs within the *PIF6* RNA reduces PTB2 binding *in vitro*, however, only Py2 is critical for AS *in vivo*.

Exon skipping by PTB1/2 depends on Py motifs within the cassette exon

In 86% of all PTB-regulated cassette exon events in *A. thaliana*, PTB1/2 mediates exon inclusion (Rühl et al., 2012). However, there are also a few PTB1/2-controlled skipping events. To see if the role of Py motifs might differ in these, we decided to investigate *AT2G34357*, which contains a cassette exon in the 5′ UTR (Figure 3a,b). Though the rate of exon skipping is low in WT seedlings (Figure 3c), there is a correlation between levels of PTB1/2 and those of the SPI variant, indicating that PTB1/2 indeed promotes skipping of this exon (Rühl et al., 2012). Splicing reporter analysis in *N. benthamiana* revealed that mutation of all candidate motifs except Py2 changed the AS ratio in the presence of the control protein (LUC, Figure S3), which

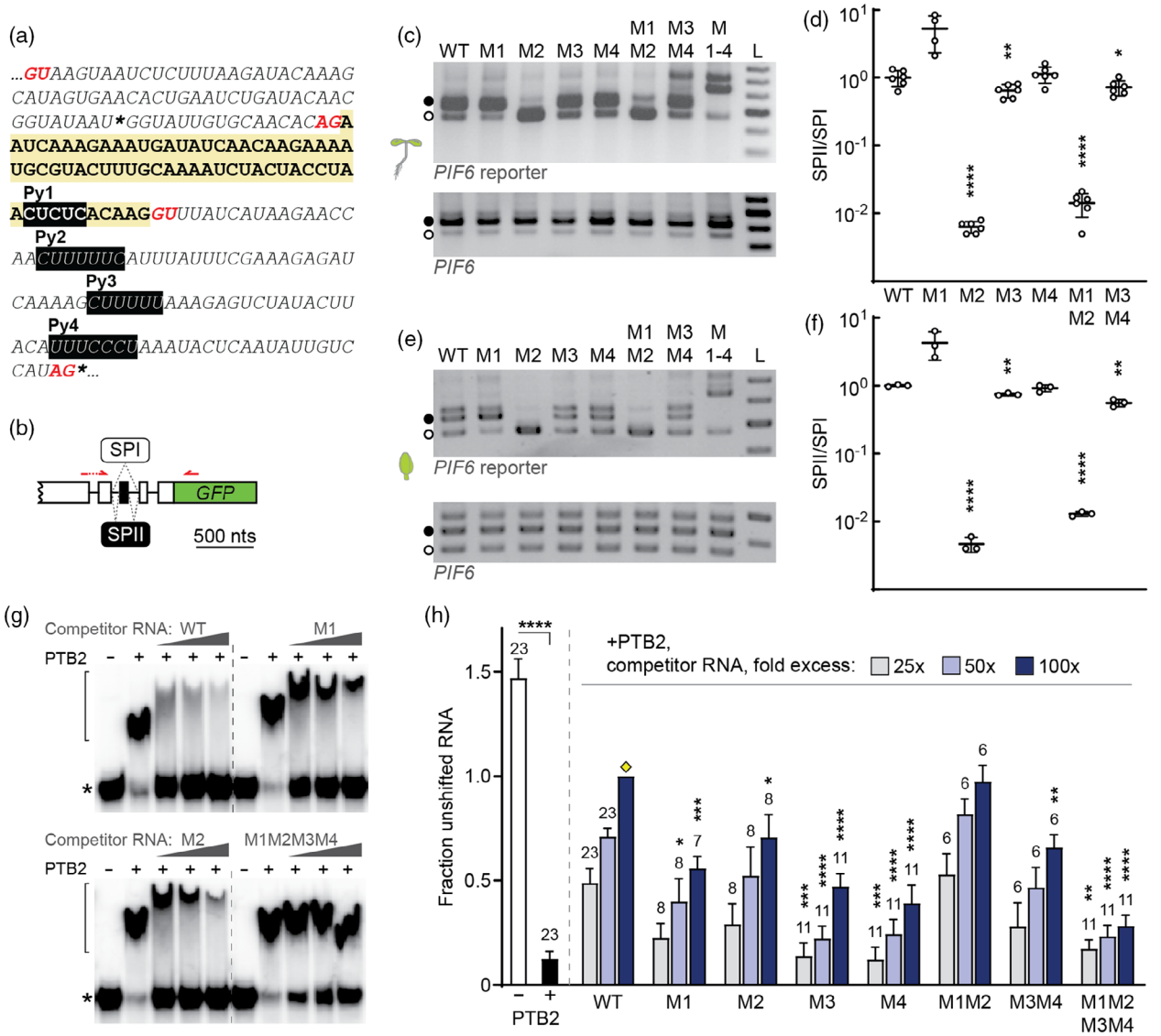

Figure 2. A single pyrimidine-rich motif is required for the inclusion of *PIF6* cassette exon *in vivo*, whereas PTB2 can bind several motifs *in vitro*.

(a) At*PIF6* sequence including the introns (italics) directly up- and downstream of the cassette exon (bold, highlighted in yellow). Splice sites (red) and pyrimidine-rich stretches (Py, black background) are highlighted. Asterisks indicate the start and end of probe used for EMSA (g, h).

(b) Splicing reporter consisting of partial At*PIF6* genomic region from start of coding region to partial second exon downstream of the cassette exon (black) fused with *GFP* cds. Alternative splicing to SPI and SPII depicted. Exons, introns, and primer binding sites shown as boxes, lines, and red arrows, respectively. Note that first exon in reporter is not fully displayed and that forward primer spans across an exon-exon border indicated by dotted region.

(c, e) Upper gel picture shows WT and mutant *PIF6* reporter splicing in seedling (c) and bud (e) samples from stable *A. thaliana* lines. Lower gel picture depicts splicing pattern of endogenous *PIF6* gene as control. L, size ladder (in 100 bp increments; stronger band corresponds to 500 bp). White and black dots indicate positions of SPI and SPII, respectively; the upper signal particularly visible for endogenous *PIF6* from bud samples likely represents a gel running artifact, as it is absent from Bioanalyzer runs and cannot be identified as distinct splicing variant using sequencing. The two larger bands detected in the M1-4 reporter sample from seedlings were identified by sequencing and result from including in addition to the cassette exon either intron 4 (amplicon length: 640 bp) or intron 3 and intron 4 (amplicon length: 718 bp).

(d, f) Quantitative analysis of reporter splicing depicted as SPII/SPI ratio normalized to the average of the WT reporter lines in seedling (d) and bud (f) samples from stable *A. thaliana* lines. Mean values (lines) with standard deviations and individual data points; asterisks indicate significant change compared to the WT reporter (one-sample *t*-test, $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$).

(g) Representative EMSA showing AtPTB2 binding to radiolabelled At*PIF6* wild type (WT) probe in the absence or presence of indicated unlabelled competitor RNAs (in 25-, 50-, and 100-fold excess from left to right). Competitor RNAs: WT sequence or mutated in indicated polypyrimidine motifs. Asterisk and bracket indicate free probe and RNA-protein complexes, respectively.

(h) Quantitative analysis of EMSA experiments. All values normalized to maximum back-shift upon competition with 100× excess of unlabelled WT RNA from the same experiment (bar marked with yellow diamond). Mean values, standard error; *n* provided above each bar; asterisks indicate significant change comparing either samples without or with PTB2 (white/black bar, unpaired *t*-test) or for the competition assays the mutant probes to the WT probe each with the same amount of competitor RNA (Dunnett's multiple comparisons test for 25× and 50×; one-sample *t*-test for 100×). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

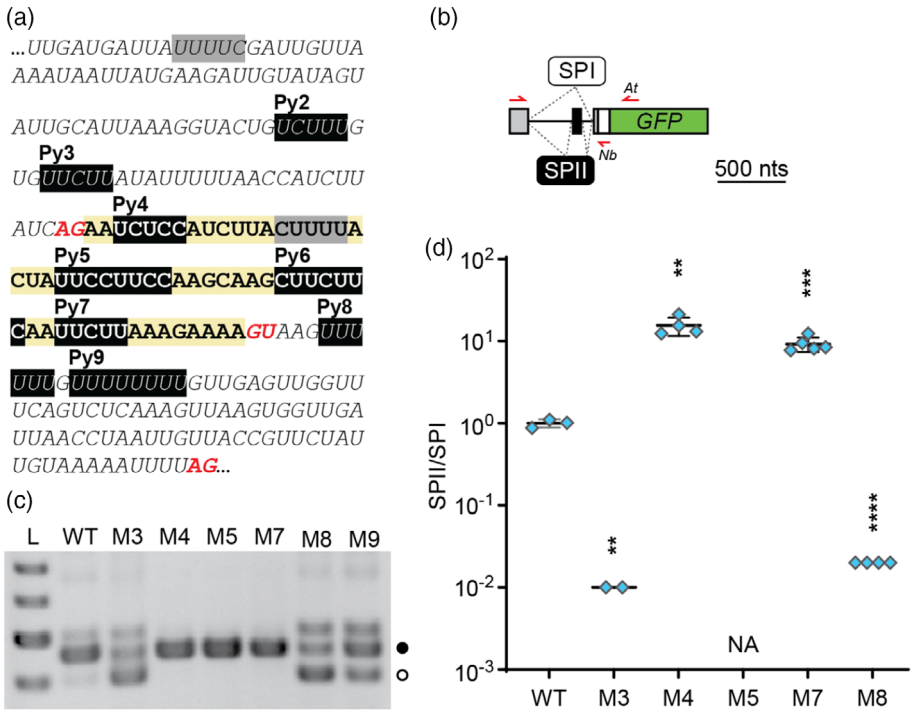

Figure 3. PTB-dependent exon skipping depends on pyrimidine-rich motifs located within the cassette exon.
(a) Partial *AT2G34357* sequence starting within the intron upstream of the cassette exon (bold, highlighted in yellow) and extending to the end of the downstream intron. Intronic sequences in italics; splice sites (red) and pyrimidine-rich stretches (Py, black/gray background) are highlighted.
(b) Splicing reporter covering *AT2G34357* genomic region from 5′ UTR (gray) to partial first coding exon (white) downstream of cassette exon (black) fused with *GFP* cds. Splicing to SPI and SPII depicted. Exons, introns, and primer binding sites shown as boxes, lines, and red arrows, respectively. The primers labeled with 'At' and 'Nb' indicate the reverse primers used in RT-PCR analysis from corresponding species.
(c) WT and mutant reporter splicing in stable *A. thaliana* lines. L, size ladder (from bottom: 0.4, 0.5, 0.6, and 0.7 kb). White and black dot indicates position of SPI and SPII, respectively. Additional band at ~500 bp is of unknown origin and is absent from Bioanalyzer runs.
(d) Quantitative analysis of SPII/SPI ratio normalized to mean value of the WT reporter lines in stable *A. thaliana* mutants. Displayed are mean value (black line), standard deviation, and individual data points; asterisks indicate significant change compared to the WT reporter (one-sample *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). NA indicates that ratio could not be determined due to complete splicing to SPII and undetectability of SPI.

may involve the action of PTB homologs from *N. benthamiana* and/or other splicing regulators. Motifs Py3 and Py9 caused an AS shift towards exon skipping when mutated, the other mutations had the reverse effect. When PTB2 was co-transformed, most mutants exhibited a similar AS response as the WT reporter; only three (M3, 8, and 9) showed a reduced AS shift. The most interesting constructs, including those with an increased SPII/SPI ratio compared to the WT reporter, were then analyzed in stable *A. thaliana* lines. Here, three motifs turned out to be involved in exon skipping, as their mutation shifted AS towards the inclusion variant: Py4, 5, and 7, all of which are located within the cassette exon (Figure 3c,d). These changes might not be completely attributable to PTB1/2, as the three motifs could have a similar function as intron markers as for example Py1 and 2 from *PTB2*. Mutation to purines would make the cassette exon appear less intronic, which might have improved splice site recognition and thus contributed to its increased inclusion in these mutants. However, later experiments indicate that at least Py4 and Py7 are involved in PTB1/2-dependent exon skipping (see next section and Figure 4g). Interestingly, three other motifs in the introns up- and downstream of the cassette exon, namely Py3, 8, and 9, seemed to have the opposite effect and promote exon inclusion, indicated by the AS shift towards the skipping variant when they are mutated (Figure 3c,d). This is also consistent with the general model that U-rich motifs can help defining intronic regions and distinguishing those from (cassette) exons. Mutation of the corresponding Py motifs makes the uridine content in the cassette exon less distinct from that in the flanking introns, which may suppress recognition of this exon and cause removal of the complete region including the introns. Whether upon reduced U-richness the complete region is spliced out as in this case or the cassette exon with flanking intronic parts is retained, as seen for the *PTB2* and *PIF6* reporter, may depend on the strength of the exonic signature of the corresponding cassette exons.

When comparing the findings from the three types of splicing reporters, it becomes obvious that the critical motifs in case of *AT2G34357* and *PIF6* are shorter than those found in *PTB2*. This could relate to the extent of

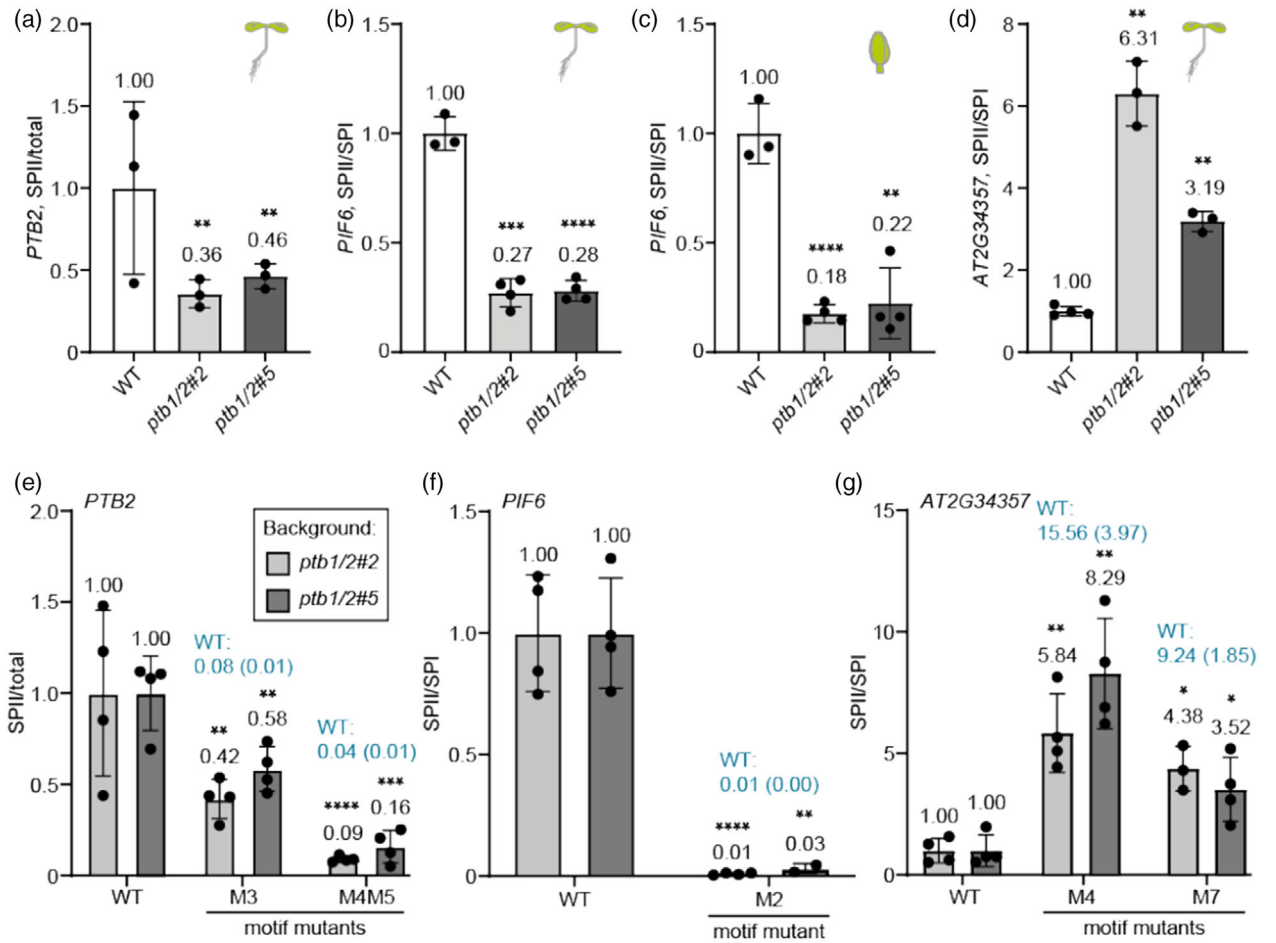

Figure 4. AS of PTB targets depends on PTB1/2 levels *in vivo*.
(a–d) Quantitative analysis of reporter splicing in seedling (a, b, d) and flower bud (c) samples of stable *A. thaliana* lines for PTB targets *PTB2* (a), *PIF6* (b, c), and *AT2G34357* (d) in WT and two independent *ptb1/2* knock-down lines. Splicing outcome is depicted as SPII/total (a) or SPII/SPI ratio (b–d) normalized to WT background. Mean values, standard deviations, and individual data points (black dots) are depicted; asterisks indicate significant change compared to the WT reporter (one-sample *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).
(e–g) Quantitative analysis of reporter splicing in seedlings of stable *A. thaliana* lines for PTB targets *PTB2* (e), *PIF6* (f), and *AT2G34357* (g) in two independent *ptb1/2* knock-down lines. Splicing outcome is depicted as SPII/total (e) or SPII/SPI ratio (f, g) normalized to the WT motif reporter in the respective mutant line. Mean values, standard deviations, and individual data points (black dots) are depicted; asterisks indicate significant change compared to the WT reporter (one-sample *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). For a direct comparison, mean values (standard deviations in parenthesis) for respective mutant reporters normalized to the WT reporter in WT background are provided in blue numbers; note that these data in the WT background are displayed in Figures 1–3.

PTB-dependent exon control. Strikingly, with the exception of *AT2G34357*-Py3, the motifs contributing to exon skipping reside within the cassette exon, while all motifs promoting cassette exon inclusion are located downstream of it, suggesting a position-dependent mode of action.

In vivo levels of PTB1/2 are coupled to the AS outcome

To be able to directly correlate PTB binding and the AS outcome *in vivo*, we decided to analyze reporter splicing for *PTB2*, *AT2G34357*, and *PIF6* in an *atptb1/2* mutant background. As a double knock-out is lethal, we used a double knock-down via an artificial microRNA (amiRNA) that targets a homologous region in the coding sequence of *PTB1* and *2* (Rühl et al., 2012). Transcript levels of *PTB1* and *PTB2* were reduced to on average approximately 36% and 54% of the WT in seedlings from two independent lines, respectively (Figure S4). This amiRNA also targets the *PTB2* reporter, however, as it is not expected that the knock-down discriminates between splice variants, quantification of splice ratios should still be valid. For all three reporters, we observed a shift towards the PTB-independent splicing variant in the amiRNA lines (Figure 4a–d). SPII levels of the *PTB2* reporter were reduced to 36% and 46% in two independent *ptb1/2* knock-down lines, respectively. In case of the *PIF6* reporter, a reduction of SPII/SPI ratio to 27% and 28% was observed. As we knew that *PIF6* splicing differs in flower buds, we also checked the effect of the mutation on reporter AS in

this tissue. It turned out that *ptb1/2* knock-down has a slightly stronger effect in flower buds than in seedlings (Figure 4c). PTB1/2 activity is therefore also required in other tissues than seedlings and might even be more limiting for correct splicing of *PIF6*. The *AT2G34357* reporter showed a three- and sixfold higher SPII/SPI ratio in mutant than in WT background, in line with PTBs' role in skipping of the corresponding cassette exon and thus elevated levels of the exon inclusion variant in the mutant lines. These observations confirm that PTB1/2 is indeed required for correct AS of these three genes.

We reasoned that if PTB1/2 is indeed binding to the aforementioned motifs *in vivo* and thereby alters the AS outcome, we should observe a lesser effect of the motif mutations in *ptb1/2* knock-down background than in WT plants. When there are no sufficient amounts of PTB1/2 present to ensure correct AS, it should hardly make a difference if binding at the target mRNA is possible or not. Therefore, the splicing shift caused by mutation of the Py motifs should be masked by the shift that appears due to the decreased PTB1/2 levels. We were able to confirm this hypothesis for two out of three events. In the WT background, SPII levels of the *PTB2* reporter drop to 8% for the M3 and 4% for the M4M5 mutation when compared to the WT reporter. In *ptb1/2* knock-down background, the decrease only amounts to an average of ~50% and ~12% between the lines, respectively (Figure 4e). Similarly, the SPII/SPI ratio of the *AT2G34357* reporter in WT background increases by factor ~16 when Py4 and factor ~9 when Py7 is mutated; in *ptb1/2* knock-down background, the ratio changes only seven- and fourfold, respectively (Figure 4g). The expected shift towards exon skipping (*PTB2*) or exon inclusion (*AT2G34357*) due to missing Py motifs is therefore less distinctive when PTB1/2 levels are low. Further evidence for *in vivo* binding of PTB2 to Py4 and Py5 in the *PTB2* pre-mRNA has recently been provided using a transient TRIBE assay (Loeser et al., 2024). In case of *PIF6*, the change in SPII/SPI ratio between WT and M2 reporters is comparable in both backgrounds (Figure 4f). This may indicate that PTB1/2 binding is not the only factor decisive for cassette exon inclusion in this gene. Alternatively, the extremely low SPII levels of the M2 reporter in the WT background might prevent resolving any further AS shift in the knockdown lines.

Motif insertion/extension is not sufficient to induce a PTB-dependent AS response

As mentioned previously, we noticed the shorter length of the critical motifs in *PIF6* and *AT2G34357* compared to *PTB2* but did not find a correlation between motif length and its effect on AS. We did see, however, that most of the stronger motifs had stretches of alternating C and U nucleotides, whereas the weaker ones (such as Py2 and Py5 in *PTB2* or Py7 in *AT2G34357*) often exhibited repeats of the same nucleotide. Because the consensus binding motif of human PTB consists of Py nucleotides with alternating C and U (Oberstrass et al., 2005; Xue et al., 2009), we wondered if we might be able to convert a weaker motif into a stronger one by adding a stretch of alternating Py nucleotides. We tested this using the *PIF6* reporter, whose only critical motif has the sequence CUUUUUC (Figure 5a). We mutated the six nucleotides directly downstream of Py2 to CUCUCU and analyzed AS in both *A. thaliana* and *N. benthamiana*. Quantification of reporter splicing in WT and *ptb1/2* knock-down plants revealed that extension of the Py2 motif did not affect the AS response to altered PTB levels in *A. thaliana* (Figure 5c). However, it caused SPII levels to decrease to about 30% compared to the WT reporter (Figure 5d). Since SPI levels did not change significantly (Figure 5e), this cannot be the result of AS. We could observe the same effects in *N. benthamiana*, although the reporter is almost exclusively spliced to SPI in this species (Figure 5f–i).

We noticed that the PTB binding motifs critical for AS are located in the intron downstream of the cassette exon for the two exon inclusion events, and within the cassette exon for the one exon skipping event (Figures 1–3). To test whether motif positioning is sufficient to define the AS outcome, we introduced PTB binding motifs at different positions into a reporter that is alternatively spliced, but not regulated by PTB1/2. As a model, we chose the *TFIIIA* gene (*AT1G72050*), containing a well-studied cassette exon event (Hammond et al., 2009) that results in similar amounts of the skipping and inclusion variant under control conditions. The critical motifs Py3 from *PTB2* and Py4 from *AT2G34357* were inserted into a previously established *TFIIIA* splicing reporter (Hammond et al., 2009). Quantification of reporter splicing in *A. thaliana* and *N. benthamiana* revealed that insertion of neither motif elicited a PTB-dependent AS response (Figure S5). Therefore, one PTB binding motif is not sufficient to make the corresponding mRNA a PTB target. This might be due to an incorrect sequence context, for example, wrong positioning of the Py stretch relative to further motifs that are recognized by the relevant splicing regulators.

Bioinformatic analysis of Py motif distribution further supports position-dependent regulation

Based on the correlation between the PTB binding position and the AS outcome in our reporter studies, we decided to raise the analysis to a more general level by analyzing Py stretch occurrence for an expanded set of PTB regulation targets. We extracted the sequences of all 81 *A. thaliana* cassette exon events that are included PTB1/2-dependently (Dataset S1; Rühl et al., 2012; Stauffer et al., 2010), and calculated the Py stretch coverage (PSC) within the alternatively spliced exons and the surrounding introns (Figure 6a–c). We compared the PSC to random samples of

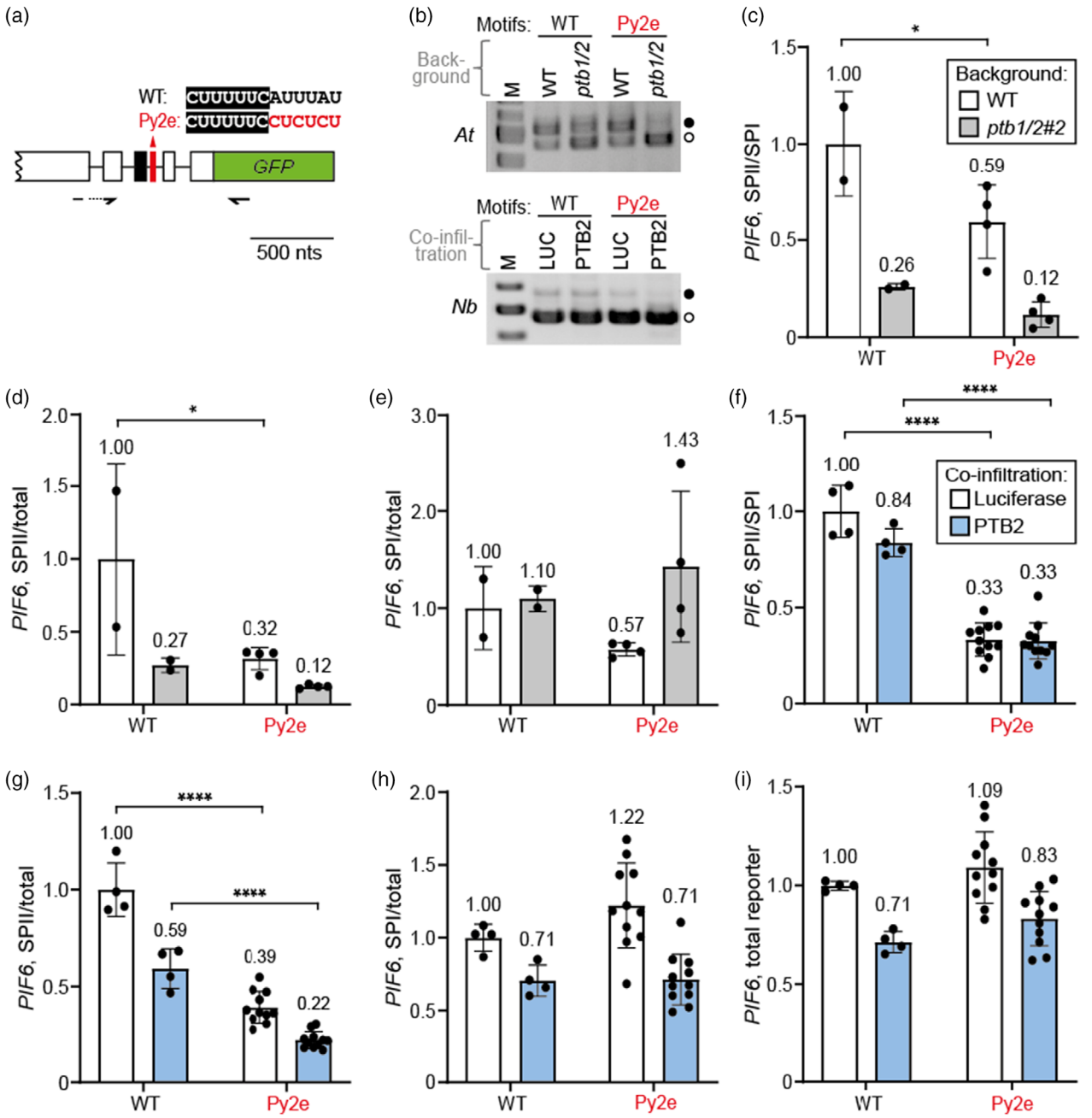

Figure 5. Motif extension has PTB-independent effects on individual transcript variants.
(a) Splicing reporter for *PIF6* with extended Py motif. Exons, introns, cds, and cassette exon are shown as boxes, lines, and white and black shading, respectively; the motif is represented by a red rectangle. Black shading of the nucleotide sequence indicates the original Py2 motif. Arrows show the approximate binding site of primers used for co-amplification [see (b)].
(b) AS pattern of the *PIF6-WT* and *PIF6-Py2e* splicing reporters in *A. thaliana* (top) and *N. benthamiana* (bottom) under varying PTB levels. M = marker, bottom to top: 400, 500, 600 bp. Black and white dots indicate splicing variants including and skipping the cassette exon, respectively.
(c–e) Quantitative analysis of reporter splicing in stably transformed *A. thaliana* lines of WT and *ptb1/2* knock-down background. Relative levels of SPI and SPII were measured and normalized to the respective WT reporter in WT background. Mean values, standard deviations, and individual data points (black dots) are depicted; asterisks indicate significant differences between the reporters in the same background (Šídák's multiple comparisons test, $*P < 0.05$).
(f–i) Quantitative analysis of reporter splicing upon transient expression in *N. benthamiana*. Relative levels of SPI, SPII, and total reporter transcript were measured upon co-expression of PTB2 or a control protein (luciferase, LUC) and normalized to co-transformed *mOrange2*. Transcript levels of the respective WT reporter co-expressed with LUC were set to 1. Data was combined from three independent experiments. Mean values, standard deviations, and individual data points (black dots) are depicted; asterisks indicate significant differences between the reporters co-infiltrated with the same protein (Šídák's multiple comparisons test, $****P < 0.0001$).

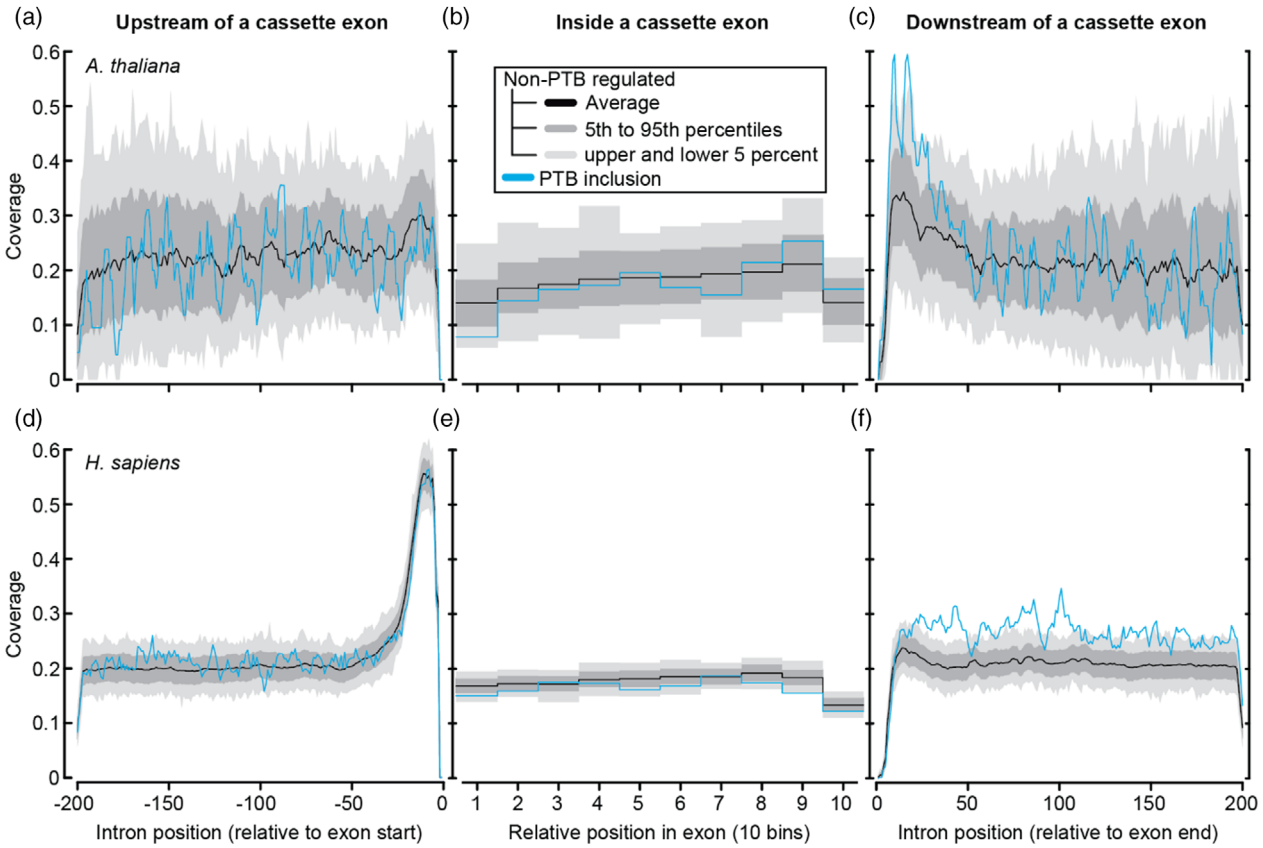

Figure 6. Pyrimidine-rich motifs are overrepresented in introns downstream of PTB-induced cassette exons.
(a–f) Pyrimidine stretch coverage was computed as the fraction of sequences that have a stretch of at least four consecutive Py nucleotides, with at least one uracil and one cytosine, at a given position for cassette exons from *A. thaliana* (a–c) and human (d–f). Blue line depicts the observed Py stretch coverage upstream (a, d), within (b, e), and downstream (c, f) of the cassette exons that are included in a PTB-dependent manner. This is compared to 1000 random samples of the same size from the much larger set of cassette exons that are not influenced by PTB binding. The average coverage of the 1000 samples is colored in black, the upper and lower 5 percent in light gray, and the values between the 5th and 95th percentiles in darker gray. Each cassette exon was split into ten equally long bins to normalize for the variability in exon length.

cassette exons that are not reported to be regulated by PTB1/2, as control. The PSC in the upstream intron and the cassette exon did not differ much between the PTB-included exons and the control. In the downstream intron, however, the PTB-regulated events show a peak in PSC within the first ~30 nt downstream of the cassette exon that exceeds the highest observed PSC value of all 1000 samples from the control (Figure 6c). This corresponds well with the location of Py2 in *PIF6*, and would also include the first of the four critical motifs within *PTB2*. The same analysis of PSC was performed for PTB-regulated exons from *Homo sapiens*, which we selected based on a previous AS study (Dataset S1; Gueroussov et al., 2015). PTB-dependent inclusion from a total of 542 events in human correlated with an altered PSC signature also in the downstream intron, as reflected by a generally higher PSC not only within 30 nt but across the whole 200 nt that we analyzed (Figure 6d–f). We also observed a substantially higher PSC directly upstream of the 3′ splice site in human compared to *A. thaliana* (Figure 6a,d). This feature is, however, independent of PTB regulation and probably corresponds to the polypyrimidine tract, which is generally less conserved in plant introns (Brown, 1986; Reddy, 2007).

Next, we examined PSC for the PTB-regulated exon skipping events. Given the small sample size of only 13 events for *A. thaliana*, the PSC showed substantial variation (Figure S6a–c). Still, we could observe an above-average PSC within the cassette exon for the PTB targets compared to the control. This pattern became even more distinct for the PTB-repressed exons from human, for which 525 events were included in our analysis (Figure S6d–f). Moreover, a much higher PSC was seen within the first ~100 nt of the intronic region upstream of PTB-repressed exons in human (Figure S6d). These signatures of PTB-regulated events in human are consistent with a previous report analyzing PSC for a smaller set of PTB-regulated events (Llorian et al., 2010). Our analysis of the larger set of human AS events additionally revealed that

PSC at the beginning of the intron downstream of PTB-repressed cassette exons is also increased (Figure S6f). Accordingly, at least some of these events might involve PTB binding up- and downstream of the exon to induce its skipping. Analyzing PSC in the extended regions of the flanking introns illustrated an increase at the beginning of the intron upstream of cassette exons from *A. thaliana* (Figure S7). Interestingly, this was seen for all cassette exons from *A. thaliana*, independent of PTB regulation, whereas introns upstream of constitutive exons did not show this feature. Similar patterns were seen when comparing the up- and downstream intron sequences from human, albeit the differences in coverage were overall less pronounced.

As PTB1/2 is not limited to the regulation of cassette exons in *A. thaliana* (Rühl et al., 2012), we investigated if there are also specific PSC signatures associated with other types of AS events. PTB1/2 from *A. thaliana* have been implicated in intron splicing/retention, alternative 5′ and also alternative 3′ splice site choice, though the latter is based on only few events and may represent mostly background. For the other two types, as already observed for cassette exons, the two proteins have a clear preference regarding the AS outcome: Most of the targeted introns are removed PTB-dependently, and PTB1/2 preferentially encourages usage of the upstream over the downstream 5′ splice site (Figures S8 and S9). Interestingly, we always found increased PSC within the ~30 nt window downstream of the alternative 5′ splice site whose usage is promoted by PTB1/2 in the respective event. In contrast, there was a depletion of Py stretches at the same position around the 5′ splice site not stimulated by PTB1/2 (Figures S8 and S9). We could detect no specific PSC signatures around alternative 3′ splice sites, neither in intron retention nor in alternative 3′ splice site choice events (Figures S8 and S10). Similarly, there were no substantial changes within the flanking exons. Taken together, PTB1/2 seems to act preferentially by defining the usage of alternative 5′ splice sites in *A. thaliana*, independent of the type of AS event, whereas human PTB might also bind close to alternative 3′ splice sites at least during cassette exon regulation.

DISCUSSION

AS is a key factor in shaping the transcriptome for adaptation to developmental or environmental cues. To be able to predict and possibly engineer these responses, we need to know the factors that control AS and understand their mode of action. Although the majority of experiments have been conducted in mammals, some conserved and recurring principles of the mechanisms of AS regulation are emerging (Brooks et al., 2011; Gueroussov et al., 2017; Koterniak et al., 2020; Reddy et al., 2013). Therefore, detailed studies of individual RBPs in different species are required to build an understanding of the underlying splicing code.

AS control by PTB1/2 from *A. thaliana* depends on Py motifs

Here, we report several insights into the mechanism of AS control by PTB homologs from *A. thaliana*. We show that cassette exon splicing by PTB1/2 depends on Py-rich motifs in the alternative exon or the flanking introns, which corresponds to the binding sequence preferences of human PTB (Pérez et al., 1997; Singh et al., 1995; Xue et al., 2009). Not all of the candidate binding motifs in this area turned out to be critical for splicing of the cassette exon, however, PTB2 also interacts with non-critical ones *in vitro*. Plant PTB homologs have been shown to be able to exert also a splicing-independent form of regulation on their targets, possibly via translation repression (Chen et al., 2022; Stauffer et al., 2010). Similarly, their mammalian counterparts play a role in multiple RNA-related processes besides AS control, such as translation initiation, mRNA processing, or degradation (Castelo-Branco et al., 2004; Fritz et al., 2020; Ge et al., 2016; Kim et al., 2010). These alternative functions might involve binding to a different set of motifs than during AS regulation.

We could not define a sequence property of AS-critical motifs that differentiates them from non-critical ones. However, most sequences from the former group contain a stretch of alternating CU nucleotides. Oberstrass et al. (2005) reported that human PTB prefers poly(CU) sequences, and a CLIP-seq experiment showed such motifs to be overrepresented among PTB binding sites (Xue et al., 2009). However, extension of a critical Py motif by a stretch of alternating Pys did not alter the AS outcome in response to PTB (Figure 5). Instead, it only affected the abundance of the exon inclusion variant, which might be caused by its increased turnover, for example, due to an incorrect assembly of spliceosome components.

Our splicing reporter analysis also indicated that Py motifs can be important to define a region as an intron. Mutation of certain Py stretches within *PTB2* and *PIF6* led to partial or complete intron retention (Figure 1d; Figure S1d; Figure 2c,e), suggesting that the higher purine content allowed this sequence to pass as exonic. Plant introns are enriched in uridines compared to exons (Dataset S1, constitutive exons), and U-, particularly AU-rich elements are important for efficient splicing (Gniadkowski et al., 1996; Ko et al., 1998; Lou et al., 1993), possibly through binding by RBPs that promote splice site recognition (Huang et al., 2022). In line with this, we found that introns flanking cassette exons are especially U-rich, probably to distinguish them from the also U-rich cassette exons (Dataset S1). Some of the Py motifs (e.g., *PTB2*-Py2 or *AT2G34357*-Py4) seem to act simultaneously as PTB binding sequence and intron signal, others fulfill only one

of these functions. Such a dual role was previously described for a plant U-rich element which can be recognized as polypyrimidine tract or intron signal depending on sequence context (Simpson et al., 2004). Interestingly, in all three genes we investigated, there are AU-rich elements in close proximity to Py motifs involved in AS: *PTB2*-Py5, *PIF6*-Py2, and *AT2G34357*-Py3 (Figures 1–3). PTB1/2 could therefore compete with other RBPs for binding at the Py motifs and/or interact with proteins bound at neighboring elements to define intron boundaries.

Arabidopsis PTBs show position-dependent control of cassette exon splicing

Our bioinformatic analysis of all known PTB1/2-regulated cassette exons and the reporter studies in *A. thaliana* suggest that the binding position of these two splicing regulators is linked to the AS outcome of the event. PTB-dependent exon inclusion is associated with an overrepresentation of Py motifs in the downstream intron, whereas PTB-regulated exon skipping events have a higher PSC within the exon. We failed to engineer a corresponding PTB-dependent AS response by inserting Py motifs at different positions into a splicing reporter (Figure S5), although such an approach has been successful in human cells (Llorian et al., 2010; Xue et al., 2009). A possible reason could be that the gene our reporter is based on is not regulated by PTB1/2, whereas the previous studies used PTB targets for their construct design. Accordingly, our modified reporters contain no Py motifs within 40 nt up- and downstream of the cassette exon, and, besides the inserted motif, only two Py stretches of 6 nt inside the exon. Most of the current models describing the regulatory mechanism of PTB homologs involve simultaneous binding of multiple Py stretches in the vicinity of the splice sites (Auweter & Allain, 2008; Hamid & Makeyev, 2017; Spellman et al., 2007), which might have prevented PTB1/2 from acting at the modified *TFIIIA* sequence. Moreover, an incorrect positioning of the Py motif relative to other *cis*-regulatory elements might have precluded a change in AS. Nevertheless, our *in vivo* data supports a position-dependent AS regulation by PTB1/2: with the exception of *AT2G34357*-Py3, all motifs promoting exon inclusion are located downstream of the cassette exon, and all motifs critical for exon skipping reside within said exon (Figures 1–3). A similar impact of motif position has been reported for other splicing regulators from the hnRNP family such as Fox and Nova in mammals and *Drosophila* (Brooks et al., 2011; Ule et al., 2006; Yeo et al., 2009; Zhang et al., 2008), including human PTB (Llorian et al., 2010). However, a conflicting study found that PTB binding close to or within the flanking constitutive exons is associated with exon inclusion, whereas exon skipping is characterized by binding close to both ends of the cassette exon (Xue et al., 2009). Therefore, we used the same approach we employed for the *A. thaliana*

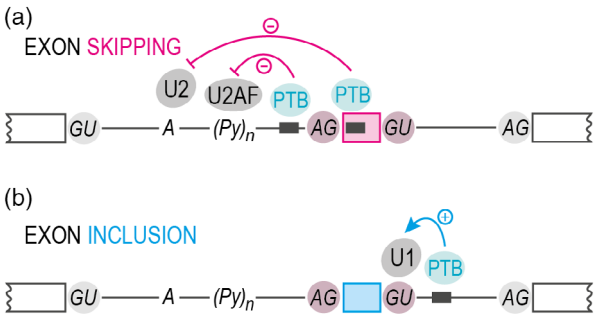

Figure 7. Model of position-dependent regulation of cassette exon skipping or inclusion.
(a) PTB binding to Py motifs (dark gray rectangles) upstream or within the cassette exon (red rectangle) results in its skipping, possibly due to interference with U2 or U2AF action at the alternative 3′ splice site.
(b) Py motif downstream of a cassette exon (blue rectangle) enhances its inclusion in a PTB-dependent manner, which can be mediated via a positive interaction with U1 at the alternative 5′ splice site.

targets to analyze Py stretch occurrence for a more recent and expanded dataset of PTB targets from human (Guerousov et al., 2015). From this dataset, 1067 PTB-regulated exons were included in our analysis, whereas the two previous studies were based on 177 (Llorian et al., 2010) and 55 (Xue et al., 2009) instances. The resulting PSC distribution supports the first model (Figures 6 and 7; Figures S6 and S7). Similar to the findings of Llorian et al. (2010) and the abovementioned studies on other hnRNPs, our computational analysis also associates PTB-dependent exon skipping in human with an increased PSC upstream of and within the cassette exon. We do not see a similar trend for the upstream intron in *A. thaliana*; this might be due to the very small sample size of only 13 PTB-dependent exon-skipping events. Interestingly, analysis of a potato invertase-based minigene reporter in tobacco protoplasts revealed that a Py motif upstream of the cassette exon was critical for AtPTB1/2-dependent exon skipping (Simpson et al., 2014). Therefore, AtPTB homologs seem to share the binding preferences linked to a specific AS outcome with their animal counterparts.

Our analysis of other types of PTB-regulated AS events (intron retention and alternative 5′ splice sites) in *A. thaliana* found elevated PSC also shortly downstream of alternative 5′ splice sites, similar as in the case of PTB-induced cassette exons. So, PTB-dependent activation of 5′ splice sites by binding within the first ~30 nt of the corresponding intron might be a common mechanism. In line with this, only the Py2 motif being positioned in the aforementioned size window was critical for PTB2-dependent inclusion of the cassette exon in *PIF6*. The window size is probably defined by the region involved in 5′ splice site recognition. Our model that 5′ splice site usage can be promoted by PTB binding to Py stretches downstream of the corresponding site is also in line with a study in mammals,

demonstrating that PTB binds to Py stretches between alternative 5′ splice sites and thereby activates usage of the upstream site (Hamid & Makeyev, 2014). The latter study also indicated regulation of alternative 3′ splice sites by human PTB, whereas in case of *A. thaliana*, very few PTB-regulated events of this type were detected (Rühl et al., 2012) and no specific PSC signatures around alternative 3′ splice sites were seen (this study). But as binding at this position could also be mediated by the polypyrimidine tract, it might not require specific PSC signatures.

Human PTB seems to require longer regions of elevated PSC than its homologs in *A. thaliana*: Whereas the area of increased PSC extends from 100 nt upstream until at least 200 nt downstream of the cassette exon in human, there is only a change within the cassette exon and the first 30 nt of the downstream intron in *A. thaliana* (Figure S7a). This could be explained by the generally longer introns in human (Figure S7b), which might require more sharply defined sequence motifs for efficient recognition by PTB. However, three out of four of the motifs critical for AS of the *PTB2* reporter are outside of the 30 nt window. Due to the autoregulation, this event is a strong target for AtPTBs (Stauffer et al., 2010; Figure S1a), which indicates that plants might also use longer regions of elevated PSC to increase regulation efficiency. It should also be noted here that the majority of the known PTB-regulated exons from *A. thaliana* require this RBP for its inclusion (Rühl et al., 2012), while mammalian PTB was primarily considered as exon silencer (Spellman & Smith, 2006). In contrast to this earlier assumption, among the more than 1000 PTB-regulated exons more recently identified in human (Gueroussov et al., 2015) the fraction of PTB-dependent skipping and inclusion is almost identical.

We also detected PSC patterns that are not necessarily coupled to a specific PTB-mediated AS outcome. Inspection of PSC at the beginning of introns provided evidence for an overrepresentation of Py motifs upstream of cassette exons compared to constitutive exons, both in *A. thaliana* and human (Figure S7). This signal in the upstream intron was similar for PTB-regulated and PTB-independent events, suggesting it might be a general feature of cassette exons. It could be linked to the regulation of this specific AS type, for example, by decelerating the splicing process and expanding the time window for a decision between cassette exon skipping or inclusion. An overrepresentation of Py motifs near both constitutive and alternative 5′ splice sites would also be in line with corresponding PTB binding sites previously identified via CLIP by Xue et al. (2009); however, this study did not examine PTB-independent cassette exons. Furthermore, PTB binding close to 3′ splice sites could be mediated via the polypyrimidine tract. In line with previous reports (Brown, 1986; Reddy, 2007; Simpson et al., 2004), we found this *cis*-regulatory element located between the branch point and 3′ splice site to be less distinct in *A. thaliana* than in human.

Taken together, general and specific signatures of PSC are associated with cassette exons and their flanking introns. Based on the PSC enrichment and our mutational studies, however, we consider the Py tracts in proximity to the cassette exons to be decisive for the PTB-regulated AS outcome.

Common binding preferences suggest that the mechanisms by which PTBs regulate AS have been conserved during evolution

The position-dependent mode of regulation is in accordance with the various reported mechanisms PTB proteins use to influence splice site choice. In fact, the binding preferences are probably an inherent consequence of these mechanisms. PTB binding downstream of the cassette exon is thought to lead to the recruitment of U1 snRNP to the alternative 5′ splice site, which would also fit to our observation of elevated PSC downstream of PTB-dependent alternative 5′ splice sites in other types of AS events (Figures S8 and S9). In contrast, binding upstream or within the cassette exon interferes with U2 recognition of the branch point and/or displaces U2AF^{65} from the polypyrimidine tract (Hamid & Makeyev, 2017; Rühl et al., 2012; Saulière et al., 2006; Stauffer et al., 2010; Zheng et al., 2014; Figure 7). Moreover, recruitment of PTB both up- and downstream of a cassette exon was reported to strongly repress its inclusion, perhaps by interfering with interactions required for exon definition (Amir-Ahmady et al., 2005; Chou et al., 2000; Hamid & Makeyev, 2017). Based on our observation that PTB-skipped exons have higher PSC not only in the upstream intron and within the cassette exon, but also at the beginning of the downstream intron, this mechanism might be more widespread in human. In addition, induction of RNA looping by simultaneous binding of two RRMs of a single PTB protein to distant Py motifs was shown in the context of exon skipping (Lamichhane et al., 2010) and would also match the aforementioned repression mechanism. Taken together, this indicates that although the preferential promotion of exon skipping or inclusion and also the pool of target genes have diverged considerably during evolution, the ancestral mechanism of AS regulation by PTB homologs probably has been retained across the animal and plant kingdoms since their separation ~1.6 billion years ago (Wang et al., 1999). Based on our findings, we conclude that PTB mainly acts via altering exon definition, making it independent of the length of introns that is on average considerably higher in human compared to *A. thaliana* (Figure S7b). In most cases, PTB is likely to act in concert with other splicing regulators whose consensus binding motifs are often quite degenerate (Goers et al., 2010; Jin et al., 2003; Llorian

et al., 2010). Interaction studies like CLIP-seq and TRIBE could be employed to confirm regulation by individual hnRNPs and establish binding positions. Thus, integrating whole-transcriptome bioinformatic analyses and detailed studies of individual AS events will bring us closer to understanding the complex interplay of regulatory decisions that shape the AS landscape.

EXPERIMENTAL PROCEDURES

Plant cultivation and transformation

A. thaliana Col-0 was grown in sterile culture. Seeds were surface-sterilized using 3.75% NaOCl and 0.01% Triton X-100 and sown onto half-strength Murashige and Skoog medium (Duchefa, www.duchefa-biochemie.com) containing 2% sucrose and 0.8% phytoagar. After stratification for 1 day at 4°C in darkness, they were transferred to a climate chamber (16 h light/22°C, 8 h dark/20°C, 60% humidity) and cultivated for 10 days before sampling or transfer to soil and further growth at the same conditions. Transformation of *A. thaliana* with reporter constructs was carried out according to the floral dip method as described by Clough and Bent (1998), either into WT background or a non-segregating line of an artificial microRNA mutant with decreased levels of *PTB1* and *PTB2* described as 'ami1&2-1' in Rühl et al. (2012). Seeds were selected by sterile culture on half-strength Murashige and Skoog medium containing 0.8% phytoagar, 50 μg ml^{-1} kanamycin for selection, and 200 μg ml^{-1} cefotaxime to prevent bacterial growth. Seeds of the following generations were grown on 50 μg ml^{-1} kanamycin. *N. benthamiana* was cultivated on soil under long-day conditions (16 h light/24°C, 8 h dark/22°C, 60% humidity in a climate chamber or under similar conditions in a greenhouse). Transient transformation was carried out according to a previously described leaf infiltration assay (Wachter et al., 2007) with the following modifications: Agrobacteria were cultivated in a YEB medium, and for the motif insertion/extension experiments, mOrange2 instead of DsRED2 was used as normalization control. Leaf tissue was sampled 2 days after infiltration for RNA extraction and 3 days after infiltration for protein isolation.

Cloning procedures

The reporters for *PTB2* (*AT5G53180*) and *TFIIIA* (*AT1G72050*) were described previously by Stauffer et al. (2010) and Hammond et al. (2009), respectively; the ones for *PIF6* (*AT3G62090*) and *AT2G34357* were constructed by PCR amplification from *A. thaliana* genomic DNA using the oligonucleotides indicated in Dataset S2. All reporters are in the pBinAR vector background (Höfgen & Willmitzer, 1992), and constructs for recombinant expression of Trx-PTB2 used pETM20 as previously described (Rühl et al., 2012). Motif mutations were introduced via overlap PCR or sub-cloning into pGEM-T (Promega, www.promega.de) and subsequent PCR mutagenesis. Detailed cloning procedures can be found in Supplemental methods. All final constructs were confirmed by sequencing the inserts. This revealed in case of the *PTB2_M4M5* mutant reporter an undesired deletion of a single C at the third nucleotide position downstream of the M5 mutation.

EMSA

EMSA and protein purification under native conditions were performed as previously described (Rühl et al., 2012). For the competition assays, unlabelled RNAs were obtained by *in vitro* transcription and gel purification as described before (Wachter et al., 2007), and mixed with the labeled probe in excess amounts (25-, 50-, and 100-fold excess) before adding the protein. Other details of reaction setup and gel analysis were as for regular EMSA without the addition of unlabelled RNAs. EMSA data were quantified using ImageQuant TL Software (GE Healthcare, www.gehealthcare.de/) to determine the fraction of the free probe.

RNA isolation and RT-PCR

Total RNA was isolated from ~100 mg plant tissue using the Universal RNA Purification Kit (Roboklon, www.roboklon.com), including an on-column DNase I treatment according to the manufacturer's instructions. Reverse transcription was performed using AMV Reverse Transcriptase Native (Roboklon, www.roboklon.com) and a dT20 primer. RT-PCR was carried out according to standard procedures, products were separated on an agarose gel and visualized by ethidium bromide staining. Quantification was performed with a Bioanalyzer 2100 (Agilent, www.agilent.com) using the DNA1000 protocol according to the manufacturer's instructions.

Quantitative PCR

Quantification of *PTB1*, *PTB2*, *AT2G34357*, *TFIIIA* and *PIF6-Py2e* transcripts took place via quantitative PCR (qPCR) using MESA-BLUE 2x qPCR MasterMix Plus for SYBR® Assay, no ROX (Eurogentec, www.eurogentec.com) on a CFX384 real-time cycler (BioRad, www.bio-rad.com). Serial dilutions of template were performed to determine primer efficiencies. All reactions were performed in triplicate, and a melting curve analysis was included. Data was analyzed using the relative standard curve method. Expression was normalized to the housekeeping gene *AT1G13320* (PP2A catalytic subunit) or to *GFP* (total reporter levels). For analysis of leaf infiltration samples, co-expressed *mOrange2* was included as additional normalization control.

Quantitative fluorescence measurement

Total protein was extracted from ~100 mg plant tissue by suspension in buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween-20, 0.1% (v/v) β-mercaptoethanol] and centrifugation at ~17 000 ***g*** and 4°C for 15 min. One hundred microliters of supernatant were transferred to a 96-well plate (black, flat bottom; Greiner Bio-One, www.gbo.com), and fluorescence of GFP and DsRED or GFP and mOrange measured using a fluorometer (Py motif mutations: TriStar, Berthold, www.berthold.com; Py motif insertions/extension: Infinite M1000, Tecan, www.tecan.de). GFP was excited at 478–492 nm and detected at 515–525 nm, DsRED/mOrange were excited at 525–535 nm and detected at 595–605 nm. To determine inhibition of mutant relative to WT reporter (Figure 1c), values upon PTB2 co-expression were normalized to LUC samples from corresponding leaf halves, the resulting value subtracted from 1 to obtain the inhibition, and then these values of the mutant reporter construct normalized to the WT reporter (i.e., the WT reporter equals a relative inhibition of 1).

Statistical analysis

Statistical analyses were performed with GraphPad Prism (GraphPad Software, www.graphpad.com) and are described in the legends and Dataset S3.

Computational analysis of Py stretch coverage (PSC)

A Py stretch was defined as a sequence of at least four consecutive Py nucleotides that contains at least one U and one C. The

PSC for a set of sequences corresponds to the fraction of sequences containing a Py stretch at each position. Exons and flanking introns were analyzed for PSC. Each exon was divided into 10 bins of equal length to normalize for variability in length. Accordingly, the PSC was also averaged for each bin in the exons. PTB-regulated and control exon sets from *A. thaliana* and human were derived from Rühl et al. (2012) and Gueroussov et al. (2015), respectively (see Dataset S1). The cassette exons being part of PTB1/2 feedback regulation (Stauffer et al., 2010) were added to the list of PTB-induced exons in *A. thaliana*. Mutually exclusive exons, that are differentially regulated by PTB, were removed from the human dataset. Further details can be found in Supplemental methods.

AUTHOR CONTRIBUTIONS

Conceptualization: AW and RB; Investigation: RB, DL, CH, MS, GW; Writing: RB and AW; Supervision: AW and ZW; Funding acquisition: AW and ZW.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

All relevant data are included in the main section or the Supporting information of the article.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Regulation of *AtPTB2* splicing reporter in *N. benthamiana*.

Figure S2. Specificity of *PIF6*-PTB2 interaction.

Figure S3. Splicing of *AT2G34357* reporter in *N. benthamiana*.

Figure S4. Efficiency of *ptb1/2* knock-down.

Figure S5. Motif insertion has PTB-independent effects on reporter abundance.

Figure S6. Distribution of pyrimidine-rich motifs in and around cassette exons skipped in a PTB-dependent manner.

Figure S7. Extended analysis of pyrimidine-rich motifs around cassette exons.

Figure S8. Distribution of pyrimidine-rich motifs around PTB-dependent introns.

Figure S9. Distribution of pyrimidine-rich motifs around PTB-dependent alternative 5′ splice site events.

Figure S10. Distribution of pyrimidine-rich motifs around PTB-dependent alternative 3′ splice site events.

Dataset S1. Coordinates and features of splicing events.

Dataset S2. Sequences and features of oligonucleotides.

Dataset S3. Details on statistical analyses.

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