Involvement of the nuclear cap-binding protein complex in alternative splicing in *Arabidopsis thaliana*

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ABSTRACT

The nuclear cap-binding protein complex (CBC) participates in 5′ splice site selection of introns that are proximal to the mRNA cap. However, it is not known whether CBC has a role in alternative splicing. Using an RT–PCR alternative splicing panel, we analysed 435 alternative splicing events in *Arabidopsis thaliana* genes, encoding mainly transcription factors, splicing factors and stress-related proteins. Splicing profiles were determined in wild type plants, the *cbp20* and *cbp80(abh1)* single mutants and the *cbp20/80* double mutant. The alternative splicing events included alternative 5′ and 3′ splice site selection, exon skipping and intron retention. Significant changes in the ratios of alternative splicing isoforms were found in 101 genes. Of these, 41% were common to all three CBC mutants and 15% were observed only in the double mutant. The *cbp80(abh1)* and *cbp20/80* mutants had many more changes in alternative splicing in common than did *cbp*20 and *cbp*20/80 suggesting that CBP80 plays a more significant role in alternative splicing than CBP20, probably being a platform for interactions with other splicing factors. Cap-binding proteins and the CBC are therefore directly involved in alternative splicing of some *Arabidopsis* genes and in most cases influenced alternative splicing of the first intron, particularly at the 5′ splice site.

INTRODUCTION

Alternative splicing is a widespread process that generates more than one spliced mRNA isoform from the same gene and expands both transcriptome and proteome diversity. Alternative events include alternative 5′ and 3′ splice site selection, intron retention, exon skipping and mutually exclusive exon splicing (1–3). Current estimates from both experimental and bioinformatic analyses are that 30–35% of *Arabidopsis thaliana* and rice genes undergo alternative splicing (4,5), while in human up to 95% of multi-exon genes undergo alternative splicing (6). The number of alternatively spliced plant genes is still likely to be an underestimate because of the relatively low EST coverage and depth of sequencing for many plant transcripts (5). In addition, many alternative splicing events occur only in specific cells and tissues, at specific stages of development and/or under certain physiological conditions and are therefore under-represented in EST databases. In plants, intron retention is the most frequent alternative event (45–56% of *A. thaliana* alternative splicing events) (4,7–10). Alternative 3′ and 5′ splice site selection accounts for ∼22 and 10% of events, respectively, and ∼4% have both 5′ and 3′ alternatively spliced sites. Only 8% of alternative splicing events in plants involve exon skipping (inclusion/exclusion of an exon) in contrast to animals where exon skipping is the most common form of alternative splicing (58% of genes) (8,11,12). The two major consequences of alternative splicing are to increase protein diversity by the inclusion or exclusion of peptide sequences or protein domains or to modulate gene expression through the production of

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mRNA isoforms which are degraded by nonsense-mediated decay (NMD). More than 75% of alternative splicing events are within the coding sequence of the gene and can generate proteins with new structures and biological functions (2,8,13). However, in both plants and animals, a significant number of AS events in the coding regions have a premature termination codon and are the potential targets of NMD (8,14,15). In plants, ~21% of all alternative splicing events take place within the 5′ UTR (15%) or 3′ UTR (6%) which can affect transport and stability of mRNAs, create new initiation codons or polyadenylation sites, generate upstream open reading frames or shift the reading frame (7,11). Regulation of alternative splicing in plants has been reported for many genes and evolutionary conserved regulation of alternative splicing of plant SR proteins which themselves are splicing regulators points to important roles of alternative splicing in plant development (16). For example, the circadian clock RNA-binding protein, AtGRP7, autoregulates its transcript levels by binding its pre-mRNA to cause alternative splicing and generate an isoform that is turned over by NMD (17). The rice *Waxy* gene encodes a granule-bound starch synthase for which alternative splicing is temperature sensitive, potentially contributing to poor grain quality when seed maturation occurs at low temperature (18). Alternative splicing of the first intron of the isoaspartyl methyltransferase 2 gene creates different protein variants found in different subcellular compartments where they are involved in protein repair processes (19). Some transcripts can be alternatively spliced in plants in response to wounding or virus infection (20–22). Alternative splicing can also be regulated in a temperature-dependent manner, such as the *A. thaliana* SR1 splicing factor (23), or in a tissue-dependent manner, for example, tobacco *RGP* (24) and spinach and tobacco chloroplast ascorbate peroxidase transcripts (25).

The eukaryotic nuclear cap-binding complex (CBC) consists of two subunits (CBP20 and CBP80) that, as a complex, bind to the cap structure of RNA polymerase II transcripts (26). The cap and the CBC have multiple functions in mRNA biogenesis including splicing (26–31), 3′-end formation by stabilizing the interaction of the 3′-end processing machinery (32,33), nuclear export (34–37) and protection of the transcripts from nuclease degradation (38–39). Constitutive and alternative splicing is mediated by the spliceosome which is assembled on an intron in a stepwise process. An early event in spliceosome assembly is the interaction of U1snRNP with the 5′ splice site of the intron. In mammals and yeast, the CBC at the 5′-end of the pre-mRNA transcript promotes the initial interaction between U1snRNP and the 5′ splice site of the first intron in the transcript, and enhances the formation of spliced mRNAs (26–31,40–44). CBP proteins remain bound to the mRNA during the pioneer round of translation playing an essential role in mRNA quality control (45). Moreover, in mammalian cells, CBP80 recruits the NMD factor Upf1 and promotes the interaction of Upf1 with the NMD factor Upf2 (46).

The plant CBC also consists of two subunits: AtCBP20 and AtCBP80. AtCBP20 has a calculated molecular mass of 29.9 kDa and exhibits 68% identity and 82% similarity

to its human orthologue and 53% identity and 77% similarity to the yeast protein. The larger CBP80 subunit is 96.5 kDa and exhibits lower identity (28 and 22%) and similarity (50 and 42%) to its human and yeast orthologues, respectively. AtCBP20 contains a canonical RNA binding domain (RBD) and AtCBP80 contains a protein–protein and protein–nucleic acid interaction domain, MIF4G (47). Additionally, in contrast to human and yeast, AtCBP20 has a long C-terminal extension with two nuclear localisation signals (NLSs) and is actively transported to the nucleus, while AtCBP80 can reach the nucleus only in a complex with AtCBP20. This feature distinguishes the mechanism of CBP transport in plants and animals where the nuclear import of human CBC requires a bipartite NLS on CBP80 (48). Moreover, neither CBP20 nor CBP80 is involved in NMD in *A. thaliana* (49).

Both plant CBC proteins are encoded by single-copy genes on Arabidopsis chromosomes V and II, respectively (47) and T-DNA insertion mutants which disrupt either the *AtCBP20* or *AtCBP80(ABH1)* genes show identical phenotypes: slow growth and serrated leaf margins. Both mutants are abscisic acid (ABA) hypersensitive and can tolerate water deficiency much better than wild type plants (50–53). The mutants also affect flowering time and the processing and splicing of mRNA of factors involved in the regulation of flowering time in *Arabidopsis* is affected (41,54,55). The increase in the occurrence of unspliced introns demonstrates a further effect of the CBC on pre-mRNA splicing (40,41,56). Finally, the plant CBC has recently been shown to mediate the biogenesis of microRNAs (miRNA) (40,53,57). Both *cbp20* and *cbp80(abh1)* mutants have reduced miRNA levels and increased pri-miRNA levels. CBP20 and CBP80 are suggested to bind to capped pri-miRNA transcripts and play role in their processing (53). The CBC interaction with the miRNA processing machinery appears to involve SERRATE (40) and is likely to facilitate the loading of the miRNA processing machinery onto pri-miRNA, in analogy with its role in recruiting the splicing commitment complex onto pre-mRNA (40,57). The role of CBP80 in miRNA-mediated RNA silencing pathway may also be conserved in animals (58). Finally, both proteins also participate in ta-siRNA biogenesis through regulating the biogenesis of miR173 and miR390 (53).

The CBC is important in pre-mRNA splicing and other aspects of RNA processing in plants. To date, there is no evidence that the nuclear cap-binding complex is involved in alternative splicing and whether the plant CBC exerts an effect on splicing by a similar mechanism to animals—promoting spliceosome assembly on the first intron—is unknown. In this article, we have addressed the influence of the nuclear cap-binding complex on alternative splicing of 252 *A. thaliana* gene transcripts showing alternative splicing, using the T-DNA insertion knock-out mutants: the single *cbp20* and *cbp80(abh1)* mutants and the double *cbp20/80* mutant. In the cases that showed significant changes in AS, the mutants preferentially affect alternative splicing of the first intron and particularly at the 5′ splice site. Similar changes in the alternative splicing profiles of

many gene transcripts were observed in all three *cbp* mutants suggesting that the CBC is directly involved in alternative splicing of these pre-mRNAs. Interestingly, our results revealed that AtCBP80 plays a more significant role in alternative splicing than AtCBP20.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana (wild-type Columbia and *cbp* mutant) seeds were placed in a bell-jar and sterilised using hydrochloric acid fumes generated from a solution containing 100 ml ACE bleach (5.25% v/v, Procter and Gamble) and 3 ml concentrated HCl. Sterilised seeds were sown in soil treated with Amistar fungicide (Syngenta), and were kept at 4°C for 72 h in the dark. After vernalisation, plants were grown in a growth chamber (SANYO MLR-350H) under controlled environmental parameters: 70% humidity, temperature 22°C, 16 h light/8 h dark photoperiod regime at 150–200 µE/m^2. Five-week-old plants were harvested and leaf tissue was flash frozen in liquid nitrogen and stored at −80°C. The *A. thaliana* wild-type Columbia was originally obtained from Lehle Seeds; the *cbp20* and *cbp80(abh1)* mutants were as described in Papp *et al.* (52) and Hugouvieux *et al.* (50), respectively. The *cbp20/80* double mutant was obtained by crossing *cbp20* and *cbp80(abh1)*, and the homozygous line was isolated based on PCR assays.

RNA extraction and RT–PCR

Total RNA was extracted from 100 mg, five-week-old leaves using the RNeasy Plant Mini Kit (Qiagen) and quantified spectrophotometrically at 260 nm. RT–PCR experiments were performed on total RNA isolated after DNase RQ1 treatment, according to the manufacturer's instructions (Promega). Reverse transcription by M-MLV RT (*RNase H-*) (Promega) was performed using oligo d(T)^{18} oligonucleotide as a primer. First, 5 µg of extracted RNA and 1 µl oligo d(T)^{18} (10 mM) were mixed in a total volume of 26 µl, incubated for 5 min at 65°C to melt secondary structures within the template, and cooled immediately for 10 min on ice. Subsequently, 8 µl M-MLV 5× Reaction Buffer, 1 µl M-MLV (*RNase H-*) (200 U/µl), 4 µl nucleotide mix (10 mM each dNTP) and 1 µl RNasin (40 U/µl) (Promega) were added to form a 40 µl volume reaction mix. The reaction mix was incubated for 1.5 h at 42°C and further incubated at 70°C for 10 min to inactivate the reverse transcriptase. The reverse transcription reaction was diluted to a final volume of 100 µl, and 1 µl cDNA was then aliquoted into a reaction tube along with 2.5 µl 10× PCR buffer with MgCl$_2$ (Roche), 4 µl nucleotide mix (1.25 µM of each dNTP, Promega), 0.75 µl of combined alternative splicing event-specific primers (100 µM stock) and Taq DNA Polymerase (5 U/µl, Roche). A 25-µl volume PCR reaction mix was then subjected to the standard PCR reaction: 94°C for 2 min, followed by 24 cycles of 94°C for 15 s, 50°C for 30 s, 70°C for 1 min and completed with 10 min at 72°C. We have shown previously that 24 PCR cycles is in the exponential amplification range for mRNA transcripts of

numerous genes with a range of expression levels (59). AS-specific primers were selected to amplify the expected alternative spliced mRNA isoforms, and gave RT–PCR products of sizes between 60 and 700 bp. In order to visualize the RT–PCR products, the forward primer was labelled with one of the following dyes: 6-FAM, VIC, NED or PET (Applied Biosystems). Primer sequences are given in Supplementary Table S1.

Splicing analysis

Labelled RT–PCR product (1 µl) from the RT–PCR reactions was mixed with 8.95 µl Hi Di Formamide (Applied Biosystems) and with 0.05 µl of GeneScan 500 LIZ or GeneScan 600 LIZ internal size standard (Applied Biosystems). Using an ABI3730 DNA Analyzer (Applied Biosystems), capillary electrophoresis of RT–PCR fragments was performed. Peak size and area data was analysed with *GeneMapper* or *PeakScanner* software (Applied Biosystems). RT–PCR products were accurately identified with ±1 nt resolution. The relative fluorescent peak areas for RT–PCR products with expected sizes for the alternatively spliced products were extracted, and a ratio for the AS events was calculated by dividing the value for the spliced products by the sum of the values for the alternatively spliced products. For an accurate measurement of AS ratios, three biological repeats were performed for all experiments. Mean alternative splicing efficiencies with standard errors were calculated for three separate biological repetitions. Means were compared by analysis of variance between wild type plants and the different CBP mutant lines and *P*-values generated. AS events with significant variation ($P < 0.10$) were selected.

RESULTS

Alternative splicing RT–PCR panel

The influence of nuclear cap-binding proteins was examined by analyzing 435 Arabidopsis alternative splicing events on a custom high resolution RT–PCR panel. The AS RT–PCR panel was based on that described previously which contained 89 AS events and seven controls (59). This panel was expanded to contain 428 AS events and seven controls. The AS events were in transcripts from genes encoding transcription factors (179), splicing factors (88), stress-related proteins (ABA) (51), stomatal ABA signaling (10), flowering time regulating proteins (16) and other miscellaneous proteins (91). The events were selected from either published AS events or from five different Arabidopsis/plant bioinformatics databases: ASIP (http://www.plantgdb.org/ASIP/EnterDB.php), TIGR (http://www.tigr.org/tdb/e2k1/ath1/), RIKEN (http://rarge.gsc.riken.jp/a_splicing/index.pl), ASTRA (http://alterna.cbrc.jp/) and TAIR 7.0 (http://www.arabidopsis.org/index.jsp). Seven genes were used as controls and amplified intronless regions (At5g03240, At5g60670, At3g61860), constitutively spliced introns (At3g12110, At5g13480) or a U12-dependent intron (At4g02560). Additionally, RuBisCo activase (At2g39730) is an alternative splicing control that consistently shows equal selection of two alternative 3′

splice sites in transcripts in all the tissues tested so far and was used as an alternative splicing control.

The splicing profiles were determined by RT–PCR with fluorescently labelled primers for each AS event using total RNA from wild type plants and from the single mutants: *cbp20* and *cbp80(abh1)* and the double mutant: *cbp20/80*. The double mutant has a similar phenotype to the single mutants: slow growth and serrated leaf margins, ABA hypersensitivity and greater resistance to water deficiency than wild type plants. PCR products were separated on an ABI3730 automated DNA sequencer and analysed by *GeneMapper* or *PeakScanner* software (Applied Biosystems). The ratio of the peak areas of the alternatively spliced isoforms for each gene in the wild type plant was compared to the ratio of the peak areas of products in the *cbp* mutants. Means and standard errors were calculated for three separate experiments.

Alternative splicing is affected in *cbp* mutants

Of the 435 genes tested, 99 genes did not show RT–PCR products. This is most likely to be due to very low level of expression of these particular genes in the plant tissue analysed (35-days-old leaves). For 84 cases only one splicing isoform was observed. This suggested that the alternative splicing events did not occur in the tissue and developmental stage analysed or represented extremely rare events even though supported by EST/cDNA sequences. As neither of the above groups provide alternative splicing information in the tissue analysed, these events were not included in further analysis.

Of the 252 remaining different AS events 101 showed significant ($P \leq 0.10$) changes in the ratio of alternatively spliced isoforms of over 3% between wild type plants and the *cbp20*, *cbp80(abh1)* or *cbp20/80* mutants. Of these events, 41 were common to all three mutants, nine were found in both the *cbp80(abh1)* and *cbp20/80* mutants, four were common to *cbp20* and *cbp20/80*, five were common to *cbp20* and *cbp80(abh1)*, 15 were found only in the double mutant, and 6 and 21 only in the single *cbp20* and *cbp80(abh1)* mutants, respectively (Figure 1). Different types of alternative splicing events were affected in the *cbp* mutants (Table 1). The majority of the alternative splicing events involved two alternative products (Table 2) and four events involved three alternative products (Table 3). Thus, the majority of AS events showing significant changes in alternative splicing

profiles (41 events—41%) were found in all three *cbp* mutants. For example, in At5g43270, both single mutants showed a similar significant change in AS but the largest change was seen in the double mutant where the ratio of the two alternatively spliced isoforms changed from 76%:24% to 51%:49% (Figure 2A). In At1g31500, the degree of change was similar in all three mutants changing from 61%:39% to ~70%:30% (Figure 2B). In At5g02470, there were different degrees of significant changes in all three mutants but again the biggest change in the ratio of AS1:AS2 was in the double mutant (from 53:47 to 80:20%) (Figure 2C). Knocking out the CBC could potentially have a general affect on mRNA stability leading to the observed changes in alternative splicing. We therefore analysed by microarray analysis the expression levels of genes in the *cbp20* and *cbp80* mutants compared to wild-type. Across all genes, >97% showed no differences in expression and in the genes containing the 101 significantly different alternative splicing events, only one had a reduced expression level and two had increased expression (data not shown). Thus, the CBC directly influences the selection of alternative splice sites and the extent and pattern of changes varied among the AS events and the different mutants.

Alternative splicing is mainly affected in the *cbp80* and *cbp20/80* double mutants

Forty-one AS events were significantly changed in all three *cbp* mutants. Of the remaining 60 events which showed
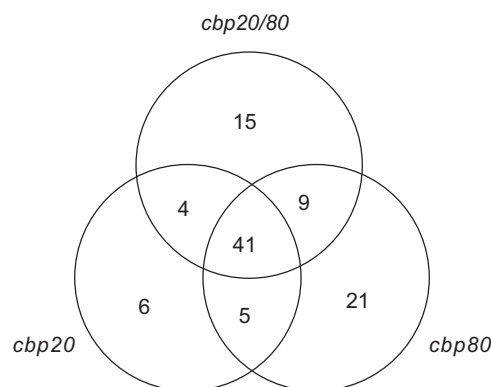
Figure 1. Distribution of alternative splicing events with significant changes in alternative splicing profiles in the *cbp* mutants.

Table 1. Distribution of different alternative splicing events with significantly changed alternative splicing profile among the *cbp* mutants

	Gene number	Alternative splicing event				
		Alt 3′ splice site	Alt 5′ splice site	Exon skipping	Alternative position	Intron retention
All *cbp* mutants	41	20	18	3	0	0
cbp80 plus *cbp20/80*	9	4	1	2	1	1
cbp20/80	15	9	3	1	0	2
cbp80	21	9	5	2	2	3
cbp20 plus *cbp80*	5	5	0	0	0	0
cbp20	6	3	3	0	0	0
cbp20 plus *cbp20/80*	4	3	0	1	0	0
Total	101	53	30	9	3	6

Table 2. Significant changes in alternative splicing isoform (two products) abundance in nuclear cap-binding complex mutants

Primer pair	Gene ID	Annotation	Intron	AS type	Location	RT–PCR product size—mRNA isoforms (bp)	Wild type—Col0	cbp20	$P \leq 0.1$	cbp80	$P \leq 0.1$	cbp20/80	$P \leq 0.1$
43	At5g09230	Trancriptional regulator (Sir2)	1	3'SS	5'UTR	157/196	0.39/0.61 ± 0.02	0.27/0.73 ± 0.02	0.0034	0.23/0.77 ± 0.01	0.0006	0.29/0.71 ± 0.03	0.008
87	At4g35450	Ankyrin-repeat-containing protein	1	5'SS	5'UTR	305/350	0.80/0.20 ± 0.01	0.93/0.07 ± 0.02	0.0017	0.94/0.06 ± 0.03	0.0009	0.95/0.05 ± 0.01	0.005
322	At2g33480	Putative NAM (no apical meristem)-like protein;COLD	2	5'SS	CDS	402/482	0.70/0.30 ± 0.06	0.81/0.19 ± 0.02	0.0981	0.85/0.15 ± 0.04	0.0274	0.82/0.18 ± 0.02	0.0603
155	At4g27050	F-box family protein	1	5'SS	5'UTR	187/350	0.07/0.93 ± 0.01	0.00/1.00 ± 0.00	0.0009	0.01/0.99 ± 0.01	0.0017	0.01/0.99 ± 0.01	0.0012
190	At5g56370	F-box family protein	1	3'SS	5'UTR	161/191	0.87/0.13 ± 0.00	1.00/0.00 ± 0.00	<0.0001	1.00/0.00 ± 0.00	<0.0001	0.95/0.05 ± 0.00	<0.0001
195	At3g01150	Polypyrimidine tract-binding protein, putative	8 (last)	5'SS	CDS	157/204	0.20/0.80 ± 0.01	0.10/0.90 ± 0.01	<0.0001	0.11/0.89 ± 0.01	0.0001	0.10/0.90 ± 0.00	<0.0001
187	At5g02470	DP-2 transcription factor, putative (DPA)	1	5'SS	5'UTR	165/294	0.53/0.47 ± 0.02	0.76/0.24 ± 0.00	<0.0001	0.67/0.33 ± 0.00	0.0006	0.80/0.20 ± 0.00	<0.0001
239	At1g31500	Endonuclease/exonuclease/phosphatase family protein	1	3'SS	CDS/5'UTR	121/136	0.61/0.39 ± 0.02	0.70/0.30 ± 0.02	0.0018	0.69/0.31 ± 0.01	0.003	0.67/0.33 ± 0.01	0.0187
270	At1g53650	RNA-binding protein	1	5'SS	CDS	177/195	0.38/0.62 ± 0.04	0.30/0.70 ± 0.01	0.0627	0.30/0.70 ± 0.03	0.0787	0.29/0.71 ± 0.03	0.0401
129	At2g40830	Zinc finger (C3HC4-type RING finger) family protein	1 (single)	5'SS	5'UTR	220/329	0.95/0.05 ± 0.00	1.00/0.00 ± 0.00	<0.0001	1.00/0.00 ± 0.00	<0.0001	1.00/0.00 ± 0.00	<0.0001
286	At1g67210	Zinc knuckle (CCHC-type) family protein	9 (last)	3'SS	CDS	175/181	0.07/0.93 ± 0.04	0.00/1.00 ± 0.00	0.0294	0.00/1.00 ± 0.00	0.0294	0.00/1.00 ± 0.00	0.0294
36	At4g12790	Unknown protein	1	3'SS	5'UTR	212/338	0.55/0.45 ± 0.01	0.46/0.54 ± 0.01	0.0001	0.46/0.54 ± 0.01	0.0002	0.49/0.51 ± 0.00	0.002
49	At5g41150	Repair endonuclease (RAD1)	5	3'SS	CDS	293/346	0.89/0.11 ± 0.01	0.75/0.25 ± 0.02	0.0002	0.80/0.20 ± 0.00	0.0024	0.78/0.22 ± 0.02	0.0007
51	At5g45510	Unknown protein	2 (last)	3'SS	CDS	117/200	0.90/0.10 ± 0.01	0.79/0.21 ± 0.04	0.0132	0.74/0.26 ± 0.01	0.0015	0.75/0.25 ± 0.03	0.0023
110	At1g72050	Zinc finger (C2H2 type) family protein	2	ES	CDS/5'UTR	202/382	0.22/0.78 ± 0.04	0.46/0.54 ± 0.04	0.0009	0.45/0.55 ± 0.03	0.001	0.43/0.57 ± 0.01	0.0018
171	At5g18620	DNA-dependent ATPase, putative	24 (last)	5'SS	CDS	213/222	0.68/0.32 ± 0.01	0.63/0.37 ± 0.00	0.0002	0.63/0.37 ± 0.01	0.0002	0.62/0.38 ± 0.00	0.0001
72	At2g04790	Expressed protein	2	5'SS	CDS	167/190	0.64/0.36 ± 0.00	0.54/0.46 ± 0.01	<0.0001	0.53/0.47 ± 0.01	<0.0001	0.54/0.46 ± 0.01	<0.0001
179	At5g48150	Phytochrome A signal transduction 1 (PAT1)	1	ES	5'UTR	204/284	0.39/0.61 ± 0.01	0.46/0.54 ± 0.01	0.0036	0.47/0.53 ± 0.02	0.0014	0.46/0.54 ± 0.00	0.0027
249	At1g72560	tRNA export mediator exportin-t	13 (last)	5'SS	3'UTR	141/150	0.32/0.68 ± 0.02	0.38/0.62 ± 0.02	0.04	0.40/0.60 ± 0.01	0.014	0.38/0.62 ± 0.03	0.0481
288	At3g12570	Expressed protein	2	3'SS	5'UTR	159/188	0.45/0.55 ± 0.00	0.60/0.40 ± 0.02	0.001	0.58/0.42 ± 0.01	0.0015	0.60/0.40 ± 0.02	0.001
324	At5g43270	Squamosa promoter binding protein-like 2 (emb|CAB56576,1);COLD	1	5'SS	5'UTR	186/270	0.76/0.24 ± 0.07	0.57/0.43 ± 0.03	0.0265	0.61/0.39 ± 0.10	0.0648	0.51/0.49 ± 0.01	0.0086
552	At4g26400	M3E9.170 (C3HC4)	1 (single)	5'SS	5'UTR	130/147	0.38/0.62 ± 0.04	0.51/0.49 ± 0.01	0.0125	0.47/0.53 ± 0.01	0.0586	0.51/0.49 ± 0.03	0.0120
21	At2g37340	RSZ33	2	3'SS	5'UTR/CDS	123/341	0.97/0.03 ± 0.00	0.93/0.03 ± 0.01	0.0585	0.89/0.11 ± 0.02	0.0024	0.91/0.09 ± 0.01	0.0087
3	At1g09140	SF2/ASF-like splicing modulator (SRp30)	10	3'SS	CDS/3'UTR	104/442	0.83/0.17 ± 0.00	0.79/0.21 ± 0.00	0.0165	0.74/0.26 ± 0.01	0.0006	0.76/0.24 ± 0.01	0.0029
174	At5g13220	Expressed protein	4 (last)	5'SS	CDS/3'UTR	173/226	0.77/0.23 ± 0.00	0.73/0.27 ± 0.01	0.0168	0.74/0.26 ± 0.02	0.0399	0.72/0.25 ± 0.00	0.0109
242	At1g60850	RNA pol subunit	1	3'SS	5'UTR	111/122	0.67/0.33 ± 0.01	0.63/0.37 ± 0.02	0.0452	0.63/0.37 ± 0.00	0.0481	0.59/0.41 ± 0.00	0.004
383	At2g43640	Signal recognition particle 14kDa family protein/SRP14 family protein	1	5'SS	5'UTR	166/212	0.16/0.84 ± 0.01	0.21/0.79 ± 0.02	0.0302	0.23/0.77 ± 0.01	0.0067	0.20/0.80 ± 0.01	0.0845
121	At2g18300	Basic helix-loop-helix (bHLH) family protein	4	3'SS	CDS	223/229	0.22/0.78 ± 0.00	0.12/0.88 ± 0.01	0.0004	0.12/0.88 ± 0.01	0.0003	0.26/0.74 ± 0.02	0.0862
149	At2g27230	Transcription factor-related	5 (last)	5'SS	5'UTR	208/246	0.25/0.75 ± 0.02	0.22/0.78 ± 0.01	0.0561	0.19/0.81 ± 0.01	0.0028	0.20/0.80 ± 0.00	0.01
132	At2g31370	bZIP transcription factor (POSF21)	5 (last)	5'SS	3'UTR	196/207	0.91/0.09 ± 0.02	0.88/0.12 ± 0.01	0.0476	0.86/0.14 ± 0.02	0.005	0.85/0.15 ± 0.02	0.0013
105	At1g33060	No apical meristem (NAM) family protein	6 (last)	3'SS	CDS	274/286	0.22/0.78 ± 0.00	0.18/0.82 ± 0.00	<0.0001	0.17/0.83 ± 0.00	<0.0001	0.19/0.81 ± 0.00	0.0002
160	At4g14410	Basic helix-loop-helix (bHLH) family protein	1	3'SS	CDS/5'UTR	182/339	0.98/0.02 ± 0.00	0.96/0.04 ± 0.00	0.0008	0.93/0.07 ± 0.01	<0.0001	0.96/0.04 ± 0.00	0.0020
194	At3g49430	Pre-mRNA splicing factor, putative	1 (single)	ES	5'UTR	141/365	0.98/0.02 ± 0.00	0.97/0.03 ± 0.00	0.0645	0.93/0.07 ± 0.01	0.0002	0.96/0.04 ± 0.00	0.0096
169	At5g24520	Transparent testa glabra 1 protein (TTG1)	1	3'SS	CDS/3'UTR	146/152	0.24/0.75 ± 0.01	0.20/0.79 ± 0.02	0.0714	0.19/0.80 ± 0.02	0.0444	0.27/0.71 ± 0.01	0.0952
264	At5g65430	GF 14 Kappa isoform	3 (last)	3'SS	CDS	245/251	0.15/0.85 ± 0.01	0.19/0.81 ± 0.01	0.0213	0.20/0.80 ± 0.01	0.0158	0.18/0.82 ± 0.01	0.0493
91	At5g05270	Unknown	1	5'SS	5'UTR/CDS	186/237	0.96/0.04 ± 0.00	1.00/0.00 ± 0.00	<0.0001	0.99/0.01 ± 0.00	<0.0001	1.00/0.00 ± 0.00	<0.0001
538	At2g20180	Phytochrome interacting facor 3-like 5 (PILS)	1	3'SS	CDS	313/276	0.83/0.17 ± 0.01	0.86/0.14 ± 0.00	0.0237	0.87/0.13 ± 0.00	0.0043	0.87/0.13 ± 0.00	0.0056
528	At1g09060	Transcription factor transcription factor/ubiquitin-protein ligase	1	3'SS	5'UTR	186/202	0.92/0.08 ± 0.04	1.00/0.00 ± 0.00	<0.0001	0.99/0.01 ± 0.01	<0.0001	0.97/0.03 ± 0.00	0.0001

(continued)

Table 2. Continued

Primer pair	Gene ID	Annotation	Intron	AS type	Location	RT–PCR product size—mRNA isoforms (bp)	Wild type—Col0	cbp20	P ≤ 0.1	cbp80	P ≤ 0.1	cbp20/80	P ≤ 0.1
142	At3g12250	bZIP family transcription factor	1	3'SS	5'UTR	203/236	0.11/0.89 ± 0.03	0.07/0.93		0.05/0.95 ± 0.01	0.0665	0.05/0.95 ± 0.01	0.0478
102	At1g27370	Squamosa promoter-binding-like 10 (SPL10)	1	3'SS	5'UTR	163/191	0.84/0.16 ± 0.01	0.86/0.14 ± 0.01		0.87/0.13 ± 0.01	0.0434	0.91/0.09 ± 0.00	0.0026
181	At5g05550	Expressed protein, similar to 6b-interacting protein 1	1 (single)	ES	CDS/3'UTR	210/308	0.66/0.34 ± 0.00	0.68/0.32 ± 0.01	0.0078	0.62/0.38 ± 0.01		0.58/0.42 ± 0.01	0.0001
284	At4g33060	Peptidyl-prolyl cis-trans isomerase	5	ES	CDS	206/293	0.90/0.10 ± 0.00	0.88/0.12 ± 0.02	0.0216	0.85/0.15 ± 0.02		0.86/0.14 ± 0.00	0.0712
513	At4g34000	Abscisic acid responsive element-binding factor 3 (ABRF3)	2	Alt P	CDS	250/269	0.02/0.98 ± 0.01	0.05/0.95 ± 0.02	0.0949	0.11/0.89 ± 0.03		0.08/0.92 ± 0.02	0.0676
145	At3g17609	bZIP transcription factor family protein	1	3'SS	CDS	151/193	0.03/0.97 ± 0.00	0.03/0.97 ± 0.01	0.0002	0.00/1.00 ± 0.00	0.0002	0.00/1.00 ± 0.00	0.0002
143	At3g12250	bZIP family transcription factor protein	2	5'SS	CDS	220/239	0.97/0.03 ± 0.00	0.95/0.05 ± 0.00		0.93/0.07 ± 0.01	0.003	0.94/0.06 ± 0.00	0.0347
137	At3g14740	PHD finger family protein	1 (last)	3'SS	CDS	288/294	0.49/0.51 ± 0.00	0.50/0.50 ± 0.01		0.52/0.48 ± 0.01	0.0215	0.52/0.48 ± 0.01	0.0068
326	At1g69250	Nuclear transport factor 2 (NTF2) family protein/RNA recognition motif (RRM)	6	IR	CDS	147/246	0.92/0.08 ± 0.02	0.94/0.06 ± 0.01		0.96/0.04 ± 0.02	0.0571	0.96/0.04 ± 0.01	0.0911
343	At3g29160	SNF1-like protein kinase (AKin11); Dark, sugar, hypoxia	1	5'SS	5'UTR	159/307	0.52/0.48 ± 0.05	0.47/0.53 ± 0.04		0.36/0.64 ± 0.08		0.29/0.71 ± 0.09	0.0389
50	At5g43910	Unknown protein	9	3'SS	CDS	191/222	0.34/0.66 ± 0.02	0.34/0.66 ± 0.04		0.33/0.67 ± 0.02		0.25/0.75 ± 0.01	0.023
32	At3g54790	Unknown protein	3	3'SS	5'UTR/CDS	142/379	0.08/0.92 ± 0.01	0.09/0.91 ± 0.05		0.07/0.93 ± 0.01		0.03/0.97 ± 0.00	0.0865
111	At1g61660	Basic helix-loop-helix (bHLH) family protein	7 (last)	5'SS	CDS	177/186	0.77/0.23 ± 0.04	0.79/0.21 ± 0.02		0.73/0.27 ± 0.02		0.70/0.30 ± 0.02	0.0431
170	At5g28770	bZIP transcription factor family protein	1	3'SS	CDS	174/195	0.21/0.79 ± 0.00	0.19/0.81 ± 0.01		0.19/0.81 ± 0.02		0.27/0.73 ± 0.01	0.0034
378	At3g62190	DNAJ heat shock N-terminal domain-containing protein	3	3'SS	CDS	144/334	0.74/0.26 ± 0.03	0.76/0.24 ± 0.01		0.79/0.21 ± 0.06		0.62/0.38 ± 0.03	0.0559
346	At4g23260	Protein kinase family protein: COLD	1	IR	CDS	287/401	0.46/0.54 ± 0.03	0.50/0.50 ± 0.03		0.59/0.41 ± 0.12		0.28/0.72 ± 0.07	0.0578
366	At5g25610	Dehydration-responsive protein (RD22); DESSICATION; COLD	2	IR	CDS	233/601	0.97/0.03 ± 0.02	0.97/0.03 ± 0.01		0.97/0.03 ± 0.01		0.87/0.13 ± 0.06	0.0737
167	At4g18020	Pseudo-response regulator 2 (APRR2) (TOC2); COLD	4	3'SS	CDS	105/115	0.07/0.93 ± 0.00	0.09/0.91 ± 0.00		0.09/0.91 ± 0.01		0.10/0.90 ± 0.00	0.0062
527	At1g53160	SPL4 (Squamosa promoter binding protein-like 4)	1	5'SS	CDS	183/187	0.08/0.92 ± 0.01	0.06/0.94 ± 0.01		0.07/0.93 ± 0.01		0.11/0.89 ± 0.02	0.0413
370	At5g35410	CBL-interacting protein kinase 24(CIPK24)/serine/threonine protein kinase (SOS2)	8	3'SS	CDS	115/120	0.60/0.40 ± 0.02	0.60/0.40 ± 0.02		0.65/0.35 ± 0.06		0.48/0.52 ± 0.05	0.046
344	At3g29160	SNF1-like protein kinase (AKin11); Dark, sugar, hypoxia	8	3'SS	CDS	195/200	0.05/0.95 ± 0.01	0.05/0.95 ± 0.01		0.05/0.95 ± 0.01		0.09/0.91 ± 0.01	0.0559
182	At5g16820	Heat shock transcription factor 3 (HSTF3)	2 (last)	3'SS	3'UTR	233/241	0.34/0.66 ± 0.00	0.38/0.62 ± 0.00		0.34/0.66 ± 0.00		0.38/0.62 ± 0.01	0.0414
227	At4g24740	Protein kinase (AFC2)	1	ES	5'UTR/CDS	152/343	0.39/0.61 ± 0.08	0.26/0.74 ± 0.07		0.40/0.60 ± 0.09		0.28/0.72 ± 0.09	0.0947
508	At1g68920	Transcription factor unknown protein: basic helix-loop-helix (bHLH)	2	3'SS	CDS	383/386	0.07/0.93 ± 0.01	0.08/0.92 ± 0.00		0.07/0.93 ± 0.00		0.03/0.97 ± 0.03	0.0928
30	At3g53270	Unknown protein	3	3'SS	5'UTR	264/360	0.81/0.19 ± 0.01	0.74/0.26 ± 0.04	0.0372	0.71/0.29 ± 0.01	0.0077	0.78/0.22 ± 0.01	
58	At5g65050	MADS-box protein AGL31 FLM	3	3'SS	CDS	209/242	0.11/0.89 ± 0.01	0.18/0.82 ± 0.04	0.0661	0.19/0.81 ± 0.02	0.0365	0.14/0.86 ± 0.00	
135	At3g54480	SKP1 interacting partner 5 (SKIP5)	3	3'SS	CDS	227/258	0.04/0.96 ± 0.01	0.25/0.75 ± 0.05	0.0075	0.28/0.72 ± 0.07	0.0038	0.07/0.93 ± 0.01	
175	At5g56420	F-box family protein	1	3'SS	5'UTR/CDS	174/181	0.00/1.00 ± 0.00	0.13/0.87	0.0115	0.10/0.90 ± 0.06	0.0292	0.00/1.00 ± 0.00	
127	At2g43010	Phytochrome-interacting factor 4 (PIF4)	6	3'SS	CDS	213/219	0.13/0.87 ± 0.01	0.09/0.91 ± 0.01	0.0163	0.09/0.91 ± 0.01	0.0317	0.14/0.86 ± 0.00	
59	At5g66010	Putative protein	1 (single)	3'SS	CDS	105/182	0.51/0.49 ± 0.01	0.44/0.66 ± 0.05		0.36/0.64 ± 0.03	0.0094	0.45/0.55 ± 0.00	
47	At5g20250	Seed imbitition protein-like	1	3'SS	5'UTR	206/222	0.34/0.66 ± 0.01	0.38/0.62 ± 0.03		0.14/0.86 ± 0.03	0.0004	0.36/0.64 ± 0.02	
125	At2g46790	Timing of CAB expression 1-like protein (TL1)	2	5'SS	CDS/5'UTR	244/252	0.79/0.21 ± 0.02	0.78/0.22 ± 0.01		0.66/0.34 ± 0.04	0.002	0.82/0.18 ± 0.00	
196	At3g01150	Polypyrimidine tract-binding protein, putative	2	ES	CDS	175/277	0.90/0.10 ± 0.01	0.92/0.08 ± 0.01		0.84/0.16 ± 0.03	0.0461	0.89/0.11 ± 0.00	
516	At4g02640	BZO2H1 (basic leucine zipper O2 homolog 1) (bZIP)	2	3'SS	CDS	238/256	0.75/0.25 ± 0.00	0.72/0.28 ± 0.03		0.69/0.31 ± 0.01	0.0214	0.73/0.27 ± 0.01	

Table 2. Continued

Primer pair	Gene ID	Annotation	Intron	AS type	Location	RT-PCR product size—mRNA isoforms (bp)	Wild type—Col0	cbp20	P ≤ 0.1	cbp80	P ≤ 0.1	cbp20/80	P ≤ 0.1
141	At3g51880	High mobility group protein alpha	7 (last)	5'SS	CDS	204/225	0.81/0.19 ± 0.02	0.84/0.16 ± 0.05	0.0830	0.88/0.12 ± 0.04		0.74/0.26 ± 0.02	
193	At1g07350	Transformer serine/arginine-rich ribonucleoprotein, putative	4	ES	CDS/3'UTR	200/296	0.57/0.43 ± 0.03	0.58/0.42 ± 0.01	0.0008	0.40/0.60 ± 0.03		0.63/0.37 ± 0.00	
549	At4g31550	WRKY DNA-binding protein 11 (WRKY11)	1	Alt P	CDS	189/192	0.76/0.24 ± 0.02	0.79/0.21 ± 0.01	0.0958	0.80/0.20 ± 0.02		0.75/0.25 ± 0.00	
305	At1g01060	LHY late elongated hypocotyl - Myb-like DNA binding	1	IR	5'UTR	160/330	0.89/0.11 ± 0.01	0.82/0.18 ± 0.04	0.0503	0.77/0.23 ± 0.06		0.84/0.16 ± 0.02	
504	At5g62000	ARF1-binding protein	15 (last)	3'SS	3'UTR	260/263	0.52/0.48 ± 0.01	0.53/0.47 ± 0.01	0.0252	0.57/0.43 ± 0.01		0.54/0.46 ± 0.01	
502	At5g02730	NPH4 (Non-phototrophic hypocotyl)	11 (last)	Alt P	CDS	205/221	0.00/1.00 ± 0.00	0.04/0.96 ± 0.01	0.0423	0.06/0.94 ± 0.03		0.04/0.96 ± 0.02	
512	At2g46270	G-box binding factor 3	9	5'SS	CDS	268/199	0.80/0.20 ± 0.04	0.77/0.023 ± 0.00	0.0925	0.73/0.27 ± 0.03		0.75/0.25 ± 0.01	
131	At2g38880	Histone-like transcription factor (CBF/NF-Y) family protein	5 (last)	5'SS	CDS/3'UTR	312/373	0.92/0.07 ± 0.00	0.90/0.10 ± 0.01	0.004	0.89/0.11 ± 0.00		0.90/0.10 ± 0.00	
71	At1g78810	Expressed protein	3 (last)	5'SS	CDS/3'UTR	212/269	0.80/0.20 ± 0.01	0.80/0.20 ± 0.00	0.0045	0.84/0.16 ± 0.01		0.82/0.18 ± 0.01	
184	At5g23090	TATA-binding protein-associated phosphoprotein Dr1 protein, putative (DRI)	1	3'SS	5'UTR/CDS	170/211	0.92/0.08 ± 0.00	0.91/0.09 ± 0.00	0.0216	0.89/0.11 ± 0.01		0.92/0.08 ± 0.00	
107	At1g59750	Auxin-responsive factor (ARF1)	12	3'SS	CDS	176/185	0.20/0.80 ± 0.00	0.20/0.80 ± 0.01	0.0294	0.23/0.77 ± 0.02		0.21/0.79 ± 0.00	
325	At2g47890	Putative CONSTANS-like B-box zinc finger protein;COLD	2	IR	CDS	497/575	0.99/0.01 ± 0.01	0.99/0.01 ± 0.01	0.0402	0.95/0.05 ± 0.02		0.97/0.03 ± 0.02	
373	At3g13224	RNA recognition motif (RRM)-containing protein	5 (last)	IR	CDS	267/494	0.74/0.26 ± 0.02	0.84/0.16 ± 0.02	0.088	0.85/0.15 ± 0.05		0.71/0.29 ± 0.04	
260	At3g26740	Light regulated protein	1	3'SS	CDS	196/207	0.04/0.96 ± 0.03	0.02/0.98 ± 0.01	0.0881	0.00/1.00 ± 0.00		0.02/0.98 ± 0.01	
545	At3g58710	WRKY69; transcription factor	1	3'SS	CDS	172/167	0.64/0.36 ± 0.02	0.66/0.34 ± 0.01	0.0955	0.68/0.32 ± 0.01		0.67/0.33 ± 0.01	
46	At5g19660	Subtilase family protein	2	3'SS	CDS	214/247	1.00/0.00 ± 0.00	0.89/0.11 ± 0.05	0.0078	0.98/0.02 ± 0.02		0.92/0.08 ± 0.00	0.0407
144	At3g23280	Zinc finger (C3HC4-type RING finger) family protein	7	ES	CDS	132/204	0.50/0.50 ± 0.02	0.57/0.43 ± 0.01	0.0040	0.52/0.48 ± 0.01		0.54/0.46 ± 0.01	0.0812
218	At2g30260	Small nuclear ribonucleoprotein U2B	1	3'SS	CDS	134/171	0.03/0.97 ± 0.00	0.06/0.94 ± 0.01	0.0791	0.05/0.95 ± 0.02		0.07/0.93 ± 0.02	0.0657
225	At3g53570	Protein kinase (AFC1) (AME2)	1	3'SS	5'UTR/CDS	140/179	0.14/0.86 ± 0.00	0.11/0.89 ± 0.01	0.0996	0.12/0.88 ± 0.01		0.10/0.90 ± 0.01	0.0421
148	At1g76510	ARID/BRIGHT DNA-binding domain-containing protein	1	5'SS	5'UTR	189/212	0.50/0.50 ± 0.00	0.74/0.26 ± 0.02	0.0492	0.54/0.46 ± 0.17		0.48/0.52 ± 0.03	
158	At4g32730	myb family transcription factor	1	5'SS	5'UTR	184/199	0.50/0.50 ± 0.02	0.41/0.59 ± 0.05	0.0549	0.44/0.56 ± 0.01		0.48/0.48 ± 0.01	
178	At5g67580	myb family transcription factor	1	5'SS	CDS	155/197	0.04/0.96 ± 0.00	0.01/0.99 ± 0.01	0.0005	0.02/0.98 ± 0.00		0.02/0.98 ± 0.00	
133	At2g32250	Far-red impaired responsive protein, putative	6	3'SS	CDS	178/184	0.39/0.61 ± 0.01	0.36/0.64 ± 0.00	0.0047	0.38/0.62 ± 0.01		0.37/0.63 ± 0.00	
109	At1g77080	MADS affecting flowering 1 (MAF1)	1	3'SS	CDS	118/158	0.00/1.00 ± 0.00	0.03/0.97 ± 0.01	0.0067	0.01/0.99 ± 0.00		0.01/0.99 ± 0.00	
6	At1g52500	Formamidopyrimidine-DNA glycolase	7	3'SS	CDS	171/329	0.91/0.09 ± 0.00	0.95/0.05 ± 0.01	0.0616	0.89/0.11 ± 0.02		0.94/0.06 ± 0.00	

The relative abundance of alternatively spliced isoforms is presented as a ratio of the two products for wild type, for the single mutants *cbp20* and *cbp80*, and the double mutant *cbp20cbp80* (*cbp20/80*). The standard errors derived from two or three repeat experiments are given with the ratios. Significant changes between the wild type and mutants is measured at $P \leq 0.10$. Only comparisons which show significant changes from wild type are presented with a *P*-value and are shaded grey. ES—exon skipping; 5'SS—alternative 5' splice site; 3'SS—alternative 3' splice site; AltP—events involving both alternative 5' and 3' splice sites; IR—intron retention; CDS—coding sequence; 5'UTR—5' untranslated region; 3'UTR—3' untranslated region.

Table 3. Significant changes in alternative splicing isoform abundance in nuclear cap-binding complex mutants for genes with three alternative splicing isoforms

Primer pair	Gene ID	Annotation	Intron	AS type	Location	RT–PCR product size (mRNA isoforms) (bp)	WT	cbp20	cbp80	cbp20/80	$P \leq 0.1$
168	At5g18240	myb family transcription factor	6 (last)	3'SS	CDS	153	0.34 ± 0.01	0.33 ± 0.00	0.34 ± 0.02	0.30 ± 0.01	0.087
						165	0.23 ± 0.00	0.18 ± 0.00	0.18 ± 0.01	0.22 ± 0.00	<0.001
						171	0.43 ± 0.01	0.50 ± 0.00	0.48 ± 0.02	0.48 ± 0.00	0.016
89	At4g38510	Vacuolar-type H+ATPase subunit B2	1	5'SS	5'UTR	216	0.43 ± 0.00	0.26 ± 0.03	0.23 ± 0.01	0.23 ± 0.00	<0.001
						232	0.19 ± 0.01	0.29 ± 0.01	0.33 ± 0.01	0.27 ± 0.00	<0.001
						289	0.38 ± 0.01	0.45 ± 0.04	0.44 ± 0.01	0.50 ± 0.00	0.034
118	At2g02960	Zinc finger (C3HC4-type RING finger) family protein	1	3'SS	5'UTR	197	0.44 ± 0.03	0.61 ± 0.01	0.64 ± 0.01	0.50 ± 0.02	0.001
						203	0.12 ± 0.01	0.13 ± 0.00	0.15 ± 0.00	0.12 ± 0.01	0.034
						222	0.45 ± 0.03	0.26 ± 0.00	0.21 ± 0.01	0.38 ± 0.03	0.002
10	At1g71696	Carboxypeptidase D, putative (sol1,1)	3	3'SS	CDS/5'UTR	158	0.55 ± 0.02	0.51 ± 0.03	0.37 ± 0.06	0.50 ± 0.00	0.045
						181	0.35 ± 0.01	0.36 ± 0.00	0.42 ± 0.03	0.39 ± 0.00	0.195
						301	0.10 ± 0.01	0.13 ± 0.00	0.21 ± 0.00	0.10 ± 0.00	0.009

The relative abundance of the three alternatively spliced isoforms is presented for each of the three products for wild type, for the single mutants cbp20 and cbp80, and for the double mutant cbp20cbp80 (cbp20/80). Standard errors are the result of two or three biological repeats. Significant changes in the abundance of each product is determined by comparing across the wild type and all three mutants. Significance is measured at $P \leq 0.10$. Products showing significant changes are shaded grey. 5'SS—alternative 5' splice site; 3'SS—alternative 3' splice site; CDS—coding sequence; 5'UTR—5' untranslated region; 3'UTR—untranslated region.

changes in either two out of three mutants or only in one of them (Figure 1), only six events involved the *cbp20* mutant while 21 and 15 events involved either the *cbp80(abh1)* or the *cbp20/80* mutants, respectively (Figure 1). In addition, nine events with significant changes in alternative splicing were common to the *cbp80* and *cbp20/80* mutants such that the majority of events affected in the *cbp* mutants were found in the *cbp80* and *cbp20/80* double mutants.

cbp mutants preferentially affect alternative splicing in the first intron

The CBC in animal and yeast systems promotes an efficient interaction between U1snRNP and the first intron, and enhances production of spliced mRNAs (29,30). To examine whether *Arabidopsis* nuclear cap-binding proteins also affected the first intron of transcripts, we compared the position of the introns affected by alternative splicing in the total number of events (252), the events which showed a significant change at least one of the mutants (101) (Figure 1) and those events which showed a significant change in alternative splicing in all three mutants (41). Among the 252 events showing alternative splicing on the RT–PCR panel, 107 events (42%) involved the first intron in the gene transcript, 102 (40%) involved an internal intron and 43 (17%) involved the last intron in the transcript (Figure 3). In the group of 101 AS events with a significantly different alternative splicing profile in at least one mutant 50% are alternatively spliced in the first intron, whereas 33 and 18% are alternatively spliced in the internal intron and the last intron of transcripts, respectively. Interestingly, of the 41 events which showed significant changes in alternative splicing in all three mutants, the first intron was alternatively spliced in 23 of the events (56%) (Figure 3). Only 19.5% involved internal introns and 24% involved the last intron. Thus, the percentage of alternatively spliced first and internal introns differs significantly between the total number of AS events analysed and those with significant changes in the three *cbp* mutants. The percentage of AS events involving the last intron increased in the AS events which were significantly different in all three mutants (Figure 3). These results indicate that for those genes where mutation of the CBC affected alternative splicing, the CBC is preferentially involved in alternative splicing of the first intron. In most cases (93%), the alternative first introns are located within the 5'UTRs or span the 5'UTR and coding sequence of the genes studied.

cbp mutants preferentially affect alternative 5' splice site selection

Since the CBC promotes an efficient interaction between U1snRNP and the 5' site of the first intron during constitutive splicing, we asked whether the CBC can also influence the selection between distal or proximal alternative 5' splice sites during alternative splicing. First, we compared the number of events involving alternative 5' splice site selection among all of the events showing alternative splicing (252), in those where AS profiles changed in at least one mutant (101) or in all three mutants (41).
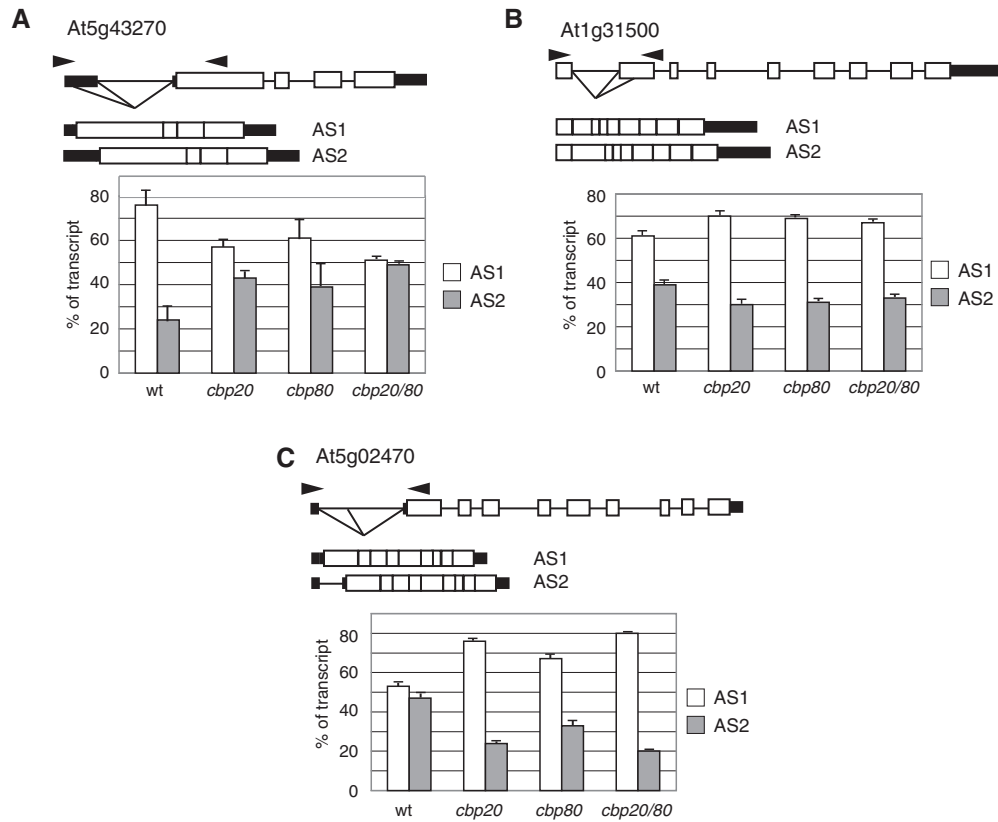
Figure 2. Gene and transcript stuctures of examples of alternative splicing events which change significantly in three *cbp* mutants. The exon/intron structure of the gene is shown with those of the alternatively spliced transcripts (AS1 and AS2). The proportion of each transcript in wild type (wt) plant and the *cbp20*, *cbp80(abh1)* and *cbp20/80* mutants is shown as a percentage in the histograms. (**A**) At5g43270, (**B**) At1g31500 and (**C**) At5g02470. Boxes—exons; black boxes—untranslated regions (UTRs); horizontal lines—introns; diagonal lines below gene structure—alternative splicing event; standard errors of the means are indicated.
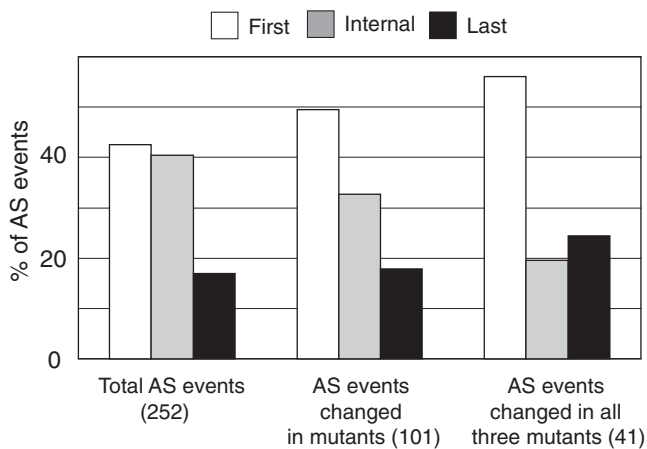
Figure 3. Percentage distribution of the position of alternative splicing events. The positions of the alternatively spliced introns (first intron, internal intron, last intron) are presented for the total events showing alternative splicing (252 AS events), AS events which changed in at least one mutant plant (101 AS events) and those which showed significant changes in all three *cbp* mutants (41 AS events).

Of the 252 AS events, 75 (30%) involved selection of alternative 5′ splice sites (Figure 4A). Similarly, of the 101 AS events changed in at least one mutant plant only 30 (30%) of AS events involved 5′ splice sites. Interestingly,

a greater proportion of the events where alternative splicing changed in all three mutants (18 of the 41 events—44%), involved alternative 5′ splice sites with fewer events involving alternative 3′ splice site selection (Figure 4A).

Second, we looked at the number of alternative 5′ splice site selection events among those involving the first intron. These groups consisted of 107 of the 252 total AS events, 50 of the 101 AS events changed in at least one *cbp* mutant and 23 of the 41 AS events with significant changes in all three mutants. Of the 107 first intron events, 36 (34%) involved alternative 5′ splice site selection. Similarly, of the 50 AS events changed in at least one *cbp* mutant, 16 (32%) involved 5′ splice site selection. Finally, of the 23 first intron events with significant AS changes in all three *cbp* mutants, 11 (48%) involved alternative 5′ splice site selection (Figure 4B). Thus, both the total number of alternative splicing events which are changed in all three mutants and those which affect the first intron are enriched in alternative 5′ splice site selection. This change in distribution suggests that CBC proteins are preferentially, but not exclusively, involved in the regulation of alternative 5′ splice site selection in the first intron.

As the CBC preferentially affects alternative 5′ splice selection in the first intron of transcripts, we examined whether the CBC also influenced the selection of the alternative 5′ splice site. If the CBC promoted usage of the 5′
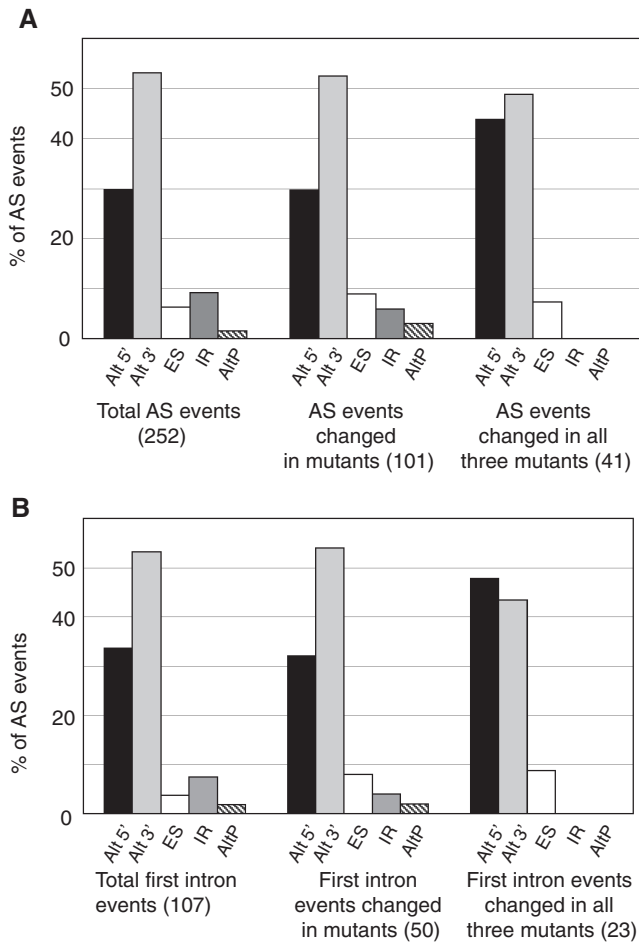
Figure 4. Percentage distribution of alternative splicing phenotypes. The alternative splicing phenotypes are presented for (**A**) the total events showing alternative splicing (252 AS events), AS events which changed in at least one mutant plant (101 AS events) and those which showed significant changes in all three *cbp* mutants (41 AS events) and (**B**) the total events involving the first intron (107 AS events), AS events changed in the first intron in at least one mutant plant (50 AS events) and those with significant changes in the three *cbp* mutants which involved the first intron (23 AS events). ES—exon skipping; Alt 5′—alternative 5′ splice site; Alt 3′—alternative 3′ splice site; AltP—events involving both alternative 5′ and 3′ splice sites; IR—intron retention.

Table 4. Use of alternative 5′ splice sites in first introns with significant changes in nuclear cap-binding complex mutants

Primer pair	Gene ID	Location	RT–PCR product size (bp)	Wild type—Col0	cbp20	P = <0.1	cbp80	P = <0.1	cbp20/80	P = <0.1	Alt 5′SS giving major isoform (in relation to cap)	Change in major isoform in mutants	CBC influence on alt 5′SS selection
87	At4g35450	5′UTR	305/350	0.80/0.20	0.93/0.07	0.0017	0.94/0.06	0.0009	0.95/0.05	0.005	Proximal	Increase	Minor (distal)
155	At4g27050	5′UTR	187/350	0.07/0.93	0.00/1.00	0.0009	0.01/0.99	0.0017	0.01/0.99	0.0012	Distal	Increase	Minor (proximal)
187	At5g02470	5′UTR	165/294	0.53/0.47	0.76/0.24	<0.0001	0.67/0.33	0.0006	0.80/0.20	<0.0001	Proximal	Increase	Minor (distal)
270	At1g53650	CDS	177/195	0.38/0.62	0.30/0.70	0.0627	0.30/0.70	0.0787	0.29/0.71	0.0401	Distal	Increase	Minor (proximal)
129	At2g40830	5′UTR	220/329	0.95/0.05	1.00/0.00	<0.0001	1.00/0.00	<0.0001	1.00/0.00	<0.0001	Proximal	Increase	Minor (distal)
324	At5g43270	5′UTR	186/270	0.76/0.24	0.57/0.43	0.0265	0.61/0.39	0.0648	0.51/0.49	0.0086	Proximal	Decrease	Major (proximal)
552	At4g26400	5′UTR	130/147	0.38/0.62	0.51/0.49	0.0125	0.47/0.53	0.0586	0.51/0.49	0.0120	Distal	Decrease	Major (distal)
383	At2g43640	5′UTR	166/212	0.16/0.84	0.21/0.79	0.0302	0.23/0.77	0.0067	0.20/0.80	0.0845	Distal	Decrease	Major (distal)
149	At2g27230	5′UTR	208/246	0.25/0.75	0.22/0.78	0.0561	0.19/0.81	0.0028	0.20/0.80	0.01	Distal	Increase	Minor (distal)
91	At5g05270	5′UTRCDS	186/237	0.96/0.04	1.00/0.00	<0.0001	0.99/0.01	<0.0001	1.00/0.00	<0.0001	Proximal	Increase	Minor (distal)
343	At3g29160	5′UTR	159/307	0.52/0.48	0.47/0.53		0.36/0.64		0.29/0.71	0.0389	Distal	Decrease	Major (proximal)
527	At1g53160	CDS	183/187	0.08/0.92	0.06/0.94		0.07/0.93		0.11/0.89	0.0413	Distal	Decrease	Major (distal)
148	At1g76510	5′UTR	189/212	0.50/0.50	0.74/0.26	0.0492	0.54/0.46		0.48/0.52		50/50	Increase (proximal)	Distal site
178	At5g67580	CDS	155/197	0.04/0.96	0.01/0.99	0.0005	0.02/0.98		0.02/0.98		Distal	Increase	Minor (proximal)
158	At4g32730	5′UTR	184/199	0.50/0.50	0.41/0.59	0.0549	0.44/0.56		0.48/0.48		50/50	Decrease (proximal)	Proximal site
89	At4g38510	5′UTR	216	0.43	0.26		0.23		0.23	<0.001	Proximal	Decrease	Major (proximal)
			232	0.19	0.29		0.33		0.27	<0.001			
			289	0.38	0.45		0.44		0.50	0.034			

The relative abundance of alternatively spliced isoforms is presented as a ratio of the two products for wild type, for the single mutants *cbp20* and *cbp80*, and for the double mutant *cbp20cbp80* (*cbp20/80*). Significant changes between the wild type and mutants is measured at P ≤ 0.10. Only comparisons which show significant changes from wild type are presented with a *P*-value and are shaded grey. CDS—coding sequence; 5′UTR—5′ untranslated region; 3′UTR—3′ untranslated region.

splice site nearest to it, these events would be expected to predominantly use the 5′ splice site proximal to the cap. A total of 16 AS events involved alternative 5′ splice sites in the first intron and showed significant changes in all three mutants (11 events) or in one mutant (five events) (Table 4). Of these, seven preferentially used the 5′ splice site closest to the cap in wild type plants, seven used the cap-distal site and two used both sites equally. This suggests that there is no preference for selection of either cap-proximal or cap-distal sites in first intron. The splicing behaviour of these events in the absence of the CBC followed two basic and subtly different patterns. In the first case, in the wild type, the CBC preferentially promoted selection of either the cap-proximal or the cap-distal site to generate more of the major alternative splicing isoform. In the knock-out mutants, the use of

these sites were reduced increasing the amount of the minor isoform. Thus, although both sites were used, the CBC preferentially used one or other of the 5′ splice sites. Six of the 16 events followed this pattern (Table 4—labelled as 'Major'). In the second case, one splice site was used preferentially in the wild-type as above, but in the mutants, use of this splice site increased showing that the CBC actively promoted usage of the alternative splice site which generates the minor splice isoform. This occurred in eight of the remaining cases (Table 4—labelled 'Minor'). In two events studied, it was impossible to point out the major splicing isoform since both alternative variants occur in equal amounts in wild type plants. However, the ratio between both splicing isoforms changed significantly in the *cbp* mutants; in one case use of the proximal 5′ss increased, and in another, use of the proximal 5′ss decreased. Our results show that there is no preference for the CBC to promote usage of the 5′ splice site closest to it, and the CBC, therefore can promote selection of one or other or both (or more) alternative 5′ splice sites to increase the levels of alternative isoforms.

Since in animals, the CBC promotes efficient splicing of the first intron during constitutive splicing we asked whether it also affects the efficiency of intron excision in plants. To address this, we looked at the six intron retention events where there was a significant change in splicing in at least one *cbp* mutant. Of these, two events involved retention of the first intron (At1g01060 and At4g23260) and two of the second intron (At5g25610 and At2g47890). Although significant in only the *cbp80* or the double mutant, all four showed a decrease in splicing of the intron. The other two intron retention events affected the fifth and sixth introns (At3g13224 and At1g69250) and splicing efficiency increased in the mutants affected. Although only a small number of intron retention events showed significant changes, those involving introns near the 5′ end of the mRNA were less efficiently spliced in at least one mutant suggesting that the CBC may influence the efficiency of removal of such introns.

DISCUSSION

The nuclear CBC in animal systems promotes an efficient interaction between the U1snRNP and the first intron of a pre-mRNA transcript, thereby enhancing spliceosome assembly and the formation of spliced mRNAs. Using knock-out mutants of the *Arabidopsis* cap-binding proteins and an alternative splicing RT–PCR panel, we have examined the influence of the CBC on alternative splicing of multiple different pre-mRNAs. The CBC significantly affected ($P \leq 0.10$) the alternative splicing of 101 events from the 252 analysed and the events involved introns at different positions in the various transcripts. However, the *cbp* mutants preferentially affected splicing within the first intron in over half of the AS events which had significant changes in the mutants. In addition, this effect was preferentially exerted at the 5′ splice site consistent with the model for animal systems that the CBC promotes 5′ splice site selection of the first intron.

Clearly many internal or last introns also showed an influence of the CBC on splicing/alternative splicing. Indeed, when considering events affected in all three mutants, the proportion of last introns affected increased as well as the proportion of first introns. The effect of the CBC on introns other than those towards the 5′-end of the mRNA may be the result of indirect regulation of other splicing factors affecting the splicing of these (and also some first introns) or may reflect three-dimensional interactions in the pre-mRNA. For example, interactions between the cap and polyadenylation proteins to circularize mRNAs increase translational efficiency (60) and have been suggested to be involved in NMD (61). In addition, plant genes and particularly introns are much smaller than those in animal genes (10) potentially permitting a range of interactions between the CBC and other regions of the transcript.

An effect of the CBC on alternative splicing has not been shown previously. Consideration of the model of the CBC promoting the interaction between the U1snRNP and 5′ splice site of the first intron predicts that, when alternative 5′ splice sites are available, the CBC would promote splicing by recruiting of U1snRNP to one or other of these sites and potentially to the 5′ splice site nearest to the cap. Our analysis of alternative 5′ splice site selection in first introns with significant changes in AS in the *cbp* mutants showed firstly that there was no preference for use of the cap-proximal alternative 5′ splice sites, and secondly, that the CBC either showed a preference for one or other site or actively promoted use of a minor site. Thus, the CBC affected usage of both major and minor alternative 5′ splice sites. This suggests that while the CBC preferentially affects alternative 5′ splice site selection in the first intron, it can promote usage of both alternative splice sites and thereby influence the levels of alternative isoforms. The choice of site and degree of use are therefore most likely to reflect the strength of the splice sites, possibly the distance of the sites from the cap, and the presence of splicing enhancer or silencer sequences and interactions of other splicing factors. This is the first demonstration that the CBC can affect alternative splicing and is again consistent with the model of the CBC mediating interactions between the U1 snRNP and 5′ splice site of the first intron.

Comparison of the effects of the two single mutants and the double mutant showed that on the basis of the number of alternatively spliced events analysed here, more AS events were affected by mutation of *AtCBP80(ABH1)* than *AtCBP20*. This suggests that the larger subunit of the plant nuclear CBC plays a more significant role in this complex during splicing/alternative splicing regulation. Given that in plants, CBP20 contains the NLSs and that nuclear import of CBP80 is thought to depend on CBP20, we expected that mutation of CBP20 would be more severe in its effects on splicing. However, although in the single mutants, no AtCBP20 or AtCBP80 is detected, the protein levels of the remaining AtCBP80 and AtCBP20 respectively vary. Interestingly, in the *cbp80(abh1)* mutant, not only is the CBP80 protein absent but also the level of AtCBP20 protein is much reduced suggesting that in the absence of AtCBP80, the

AtCBP20 subunit is unstable (48). Thus, the *cbp80* mutant is very similar to the double mutant in terms of the levels of CBP20 and CBP80 proteins and the splicing phenotypes of these mutants. In contrast, in the *cbp20* mutant which affected fewer of the alternative splicing events, AtCBP20 was absent but the AtCBP80 protein was present only at slightly reduced level to wild-type. This suggests that in the absence of AtCBP20, the larger subunit may still be able to interact in the splicing process albeit less efficiently. In yeast, CBP20-deficient (*cbp20-Δ*) mutants accumulated amounts of yCBP80 similar to those observed in the wild type strain, whereas the *cbp80-Δ* strain accumulated four-fold less yCBP20 than the wild type, suggesting that yCBP20 is unstable in the absence of yCBP80 (44,62). This observation suggests that protein interaction with CBP80 may modulate the stability of its partners. Assuming that AtCBP80 can interact with other splicing factors, it may still recruit factors to particular pre-mRNAs and thereby exert a stronger influence on splicing than AtCBP20. It was previously shown that the level of the CBC in the nucleus is precisely regulated (48) and the composition of the protein bound to the cap changed dynamically during a growth cycle in *Arabidopsis*, playing a role in the regulation of gene expression (63). Finally, that the single and double mutants are viable [similar to yeast (42,62)], and that many splicing events are unaffected in the mutants in this analysis, suggests the splicing of some introns is more dependent on the CBC than others. This may reflect the strength of intron splicing signals and of splicing enhancer sequences which can recruit splicing factors without the CBC.

Some splicing alterations were unique to the *cbp20* and the *cbp80(abh1)* mutants which may reflect variation in the degree of dependence of intron splicing on the cap or proteins associated with the CBPs or varying ability of other proteins to compensate in the absence of one or other of the CBPs. Similarly, some AS changes were observed in the double mutant only. It is also possible that loss of one or other or both CBPs affects the splicing/ alternative splicing of transcripts of genes encoding specific factors required for splicing of certain genes thereby indirectly affecting splicing of these genes. The fact that alternative splicing of transcription and splicing factors may be regulated by the CBC complex can explain, at least partially, the growth, developmental and physio-logical phenotypes of the mutants. In conclusion, in addition to constitutive splicing, the CBC is involved in alternative splicing in plants. In both cases, the CBC preferentially influences the first intron, particularly at its 5′ splice site during alternative splicing in plants.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

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